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Breast Cancer Risk - Environmental and Genetic Effects on Cancer Development, Progression and Survival

Mallin, Aimee

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Breast Cancer Risk – Environmental and Genetic Effects on Cancer Development, Progression and Survival

Aimee Mallin

Masters of Research

University of Dundee

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List of Abbreviations

7DDD	7 Day Diet Diary
BMI	Body Mass Index
cDNA	Complementary Deoxyribonucleic acid
CI	Confidence Interval
DAG	Diacyl Gylcerol
DCIS	Ductal Carcinoma in situ
DISC	Death Inducing Signalling Complex
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
CRP	C Reactive Protein
ER	Oestrogen Receptor
FFPE	Formaldehyde Fixed Paraffin Embedded Tissue
FFQ	Food Frequency Questionnaire
FFTP	First Full Term Pregnancy
FGF	Fibroblast Growth Factor
GWAS	Genome Wide Association Study
HBC	Hormonal Birth Control
hCG	Human Chorionic Gonadotrophin
HER2	Human Epidermal Factor Receptor 2
HMG	High Mobility Group
HR	Hazard Ratio
HRT	Hormone Replacement Therapy
HSPG	Heparin/Heparin Sulphate Proteogylcan
IP3	Inositol 1,4,5-triphosphate
LCIS	Lobular Carcinoma in situ
MAPK	Mitogen Activated Protein Kinase
MWS	Million Women Study
NAT2	N-acetyl Transferase 2
NHS	Nurses' Health Study
NICE	National Institute for Health and Care Excellence
NO_2	Nitrogen Dioxide
NST	No Specific Type
OCP	Oral Contraceptive Pill

OR	Odds Ratio
PAH	Polyaromatic Hydrocarbons
PR	Progesterone Receptor
RNA	Ribonucleic Acid
RR	Relative Risk
RT-PCR	Real Time Polymerase Chain Reaction
SE	Standard Error
SES	Socioeconomic Status
SHBG	Sex Hormone Binding Globulin
SNP	Single Nucleotide Polymorphism
TSP	Total Suspended Particles
WEB	Western New York Exposures and Breast Cancer Study
WHEL	Women's Healthy Eating and Living
WHI	Women's Health Initiative
WINS	Women's Intervention Nutrition Study

Risk Genes

ATM	Ataxia Telangiectasia Mutated
BARD1	BRCA1-associated RING domain protein
BRCA1	Breast Cancer Early Onset 1
BRCA2	Breast Cancer Early Onset 2
CASP8	Caspase 8
CDKN2A/B	Cyclin-Dependent Kinase Inhibitor 2 A/B
CHEK2	Checkpoint kinase 2
COX11	Cytochrome C Oxidase Assembly Protein 11
ESR1	Oestrogen Receptor 1
FGFR2	Fibroblast Growth Factor Receptor 2
LSP1	Lymphocyte Specific Protein 1
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1
NEK10	Never in Mitosis Related Kinase 10
NOTCH2	Neurogenic locus notch homolog protein 2
PALB2	Partner and Localiser of BRCA2
PDCD9	Programmed Cell Death Protein 9
PTEN	Phosphate and Tensin Homolog
RAD51	RAD51 Recombinase
RAD51L	RAD51 Homolog B
RUNX1	Runt Related Transcription Factor 1
SLC4A7	Sodium Bicarbonate Co-transporter Member 7
TNRC9	Trinucleotide Repeat Containing Gene 9
TP53	p53
ZNF365	Zinc Finger Protein 356

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Declaration

I confirm that I am the author of this thesis and that, unless otherwise stated, I have consulted all references cited personally. The work recorded in this thesis has been my own and has not previously accepted for higher degree.

Signed …….

Date ……

Breast Cancer Risk: Environmental and Genetic effects in Cancer Development, Progression and Survival

Abstract

Breast cancer is a common, complex multifactorial disease which has an estimated risk in the UK of 1 in 9 women. Due to its prevalence there has been a great deal of research carried out to identify risk and prognostic factors which are involved in its pathogenesis. By reviewing this vast array of literature conclusions have been drawn as to which factors are consistently associated with risk and prognosis. These include high penetrance genetic factors such as *BRCA1, BRCA1, PTEN;* and low penetrance mutations including *FGFR2, CASP8* and *ESR1*. Additionally environmental factors influence risk, including reproductive factors, alcohol consumption, smoking and social deprivation. Of the factors identified in this literature review there was evidence that there may be common biological mechanisms underlying their role in breast cancer risk. For example, oestrogen signalling pathways and DNA repair pathways were commonly proposed as the mechanism underlying both genetic and environmental risk.

To demonstrate the impact of environmental risk factors on breast cancer outcome a survival analysis was carried out on 1851 women diagnosed with primary breast cancer between 2000 and 2004. Using SPSS to analyse the data it was found that women from the most deprived areas in Tayside had the poorest breast cancer outcome – these outcomes included all-cause mortality, breast cancer specific death and breast cancer recurrence. When the analysis was adjusted for staging information the significant difference in breast cancer specific 5 year survival was lost. Therefore this suggests that deprived women

present with higher stage tumours, which is one of the reasons underlying their poorer outcome.

This thesis clarifies the risk and prognostic factors associated with breast cancer, and demonstrated that in a Tayside cohort deprivation is associated with poor outcome. In addition common biological mechanisms have been identified as associated with these risk factors. It is only through thinking of breast cancer in a holistic manner and incorporating different aspects of breast cancer pathogenesis a better understanding of the disease can be gained, and new risk factors identified. By understanding this better it is hoped that this will lead to improved preventative measures and the development of more targeted treatment options.

Breast Cancer Risk – Environmental and Genetic Effects on Cancer Development, Progression and Survival

Introduction

Breast cancer is the most common cancer affecting women; in 2008 there were an estimated 1.38 million cases diagnosed worldwide, with the highest incidence in Western Europe(1). It is also the most common female cancer in the UK, with a population risk of approximately 1 in 8 women(1). Due to its high prevalence there has been a great deal of research to elucidate the aetiology and optimise the management of the disease. However this has been a difficult area of research as breast cancer is a heterogeneous multifactorial disease, which involves a complex interplay between environmental and genetic factors(2).

The incidence of breast cancer has been steadily rising worldwide since the beginning of the 20th century. This increase may be due to a multitude of factors including the introduction of screening programs, environmental and societal changes. However, this has been matched by a trend towards increased expected survival over recent years which continues to increase(3). This increase in survival may be attributable to various factors including improved awareness, early detection and better clinical management.

It is also important to note that there are many forms of breast cancer, as it is heterogeneous disease which has many different pathological appearances and therefore clinical courses. These can initially be broken down into invasive or in situ carcinoma, these in situ lesions are not breast cancer they are a form of breast disease(4). There are two in situ lesions – ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). DCIS is relatively common breast disease and accounts for 20-30% of the lesions picked up by the

screening program. However approximately 35% of these DCIS cases will then go on to become an invasive carcinoma, which is why DCIS is treated in an aggressive manner despite not being a cancer. There are also various types of invasive lesions, the most common of which is ductal carcinoma, otherwise known as no specific type (NST), which accounts for about 50% of all breast cancers(5). Other varieties of breast carcinoma include lobular, tubular, medullary and mucinous carcinomas; all of which have a distinct pathological appearance.

In addition to the distinct pathological forms of the disease there are also a number of predictive factors, which include oestrogen receptor (ER), progesterone receptor (PR) and/or human epidermal factor receptor 2 (HER2) expression(6). A positive receptor status indicates that these tumours may be responsive to that particular hormone and this helps to predict patient prognosis, and informs the potential management options. These management options refer specifically to adjuvant therapies, such as tamoxifen used in ER positive tumours which targets the oestrogen signalling pathway. However it is also important to note that a positive receptor status does not mean that these tumours will definitely be responsive to these adjuvant therapies. Other management options include radiotherapy, chemotherapy and surgical management – the management plan chosen is dependent on the type, pathological characteristics and stage of tumour at diagnosis(4). However, regardless of the subtype of breast cancer there appear to be common risk factors and prognostic factors which contribute to the pathogenesis and outcome of the disease.

This thesis aims to assess the evidence for factors which contribute to breast cancer risk and prognosis. As stated above breast cancer is a complex multifactorial disease which has a variety of environmental, reproductive and genetic risk factors at play. A number of the reproductive and environmental risk factors shall be reviewed in a journalistic literature

review to assess their contribution to risk and the mechanism by which they influence risk. This shall be followed with a similar review of the genetic factors which contribute to risk, including the contribution of both high risk genetic changes and low penetrance single nucleotide polymorphisms (SNP). The environmental factors which contribute to prognosis, many of which will have been reviewed with regards to risk, shall also be investigated in the same way. This large literature review will be followed by a survival analysis of a Tayside cohort, which incorporates some of the prognostic factors previously discussed.

Due to the vast amount of literature on this subject it is not always clear what epidemiological and genetic factors contribute breast cancer development. However, the above proposed structure shall allow this thesis to review this evidence and arrive at several conclusions. This will allow for some key research questions to be answered, which will hopefully add to the current understanding of breast cancer as a multifactorial heterogeneous disease. These include:

- What environmental and genetic factors contribute to breast cancer risk and by what mechanisms?
- Do similar factors contribute to breast cancer outcome?
- Do these genetic and environmental factors interact with each other to change this contribution to risk?
- Are there common pathways which link these genetic and environmental factors?
- Finally, what is the current breast cancer survival in a small cohort? Does deprivation, one of the studied prognostic and risk factors, have any effect on breast cancer survival?

This multifactorial approach to breast cancer aims to provide a well-rounded holistic view of the numerous factors which contribute to the development, progression and outcome of breast cancer. An original survival analysis shall be utilised to gain further understanding of how these factors contribute to outcome. Due to the complex heterogeneity of breast cancer and the many elements which contribute to the disease, an understanding of the processes involved in the disease itself requires looking at these elements together and how they may interact. In the process of achieving these goals this thesis hopes to provide a comprehensive overview of breast cancer risk and prognostic factors that are commonly accepted as contributing to the disease process.

Genetic Factors Contributing to Breast Cancer Risk

1. Introduction

There are two major types of genetic factors which are known to be involved in the pathogenesis of breast cancer. The first of which are highly penetrant mutations inherited in an autosomal dominant manner; these include breast cancer 1 early onset (BRCA1) and breast cancer 2 early onset (BRCA2). The second form of inherited breast cancer risk, are low penetrance polymorphisms which confer an increased risk but are not causative of the disease. There have been 18 of these low penetrance polymorphisms identified and generally accepted as having an impact on breast cancer risk(7,8).

This literature review was conducted in a journalistic style based in part on a predefined list of breast cancer susceptibility genes, which were the first 18 SNPs and higher penetrance genes to be consistently linked with breast cancer risk. For each gene a Medline search was conducted using Breast Neoplasm and an appropriate term for the gene summarised in Appendix 1. During this review of the literature other genetic risk factors out with this pre-defined list were also identified, and therefore included in this review.

Throughout the course of this chapter the contributions of the different genetic factors to breast cancer risk and pathogenesis shall be discussed. This will also include a brief look at the risk conferred by a family history of breast cancer, followed by a discussion of the known genetic factors which contribute to breast cancer risk.

2. Family History

Family history was first noted as contributing to breast cancer risk by the French physician Paul Broca in 1866, who identified there were families with multiple cases of the

disease(9). There has been a huge amount of research in this field with the vast majority of the epidemiological work showing a positive association between family history and breast cancer risk. However relative risks have been shown to vary due to a number of factors; including the age of the individual, the nature and extent of the family history(9). The increased risk associated with family history will be due to genetics, and also in part to shared environments and social factors.

A large meta-analysis was carried out in 1997 by Pharoah et al(9), which assessed 74 studies to identify the relationship between family history and risk in a large population. They found that the results tended to remain similar regardless of study design (e.g. prospective, retrospective cohorts, case-control and hospital case-control), with RR (relative risk) ranging from 1.6 to 2.5 for those with affected first degree relatives. These results can be considered to represent the true relationship between family history and breast cancer risk, due to their homogeneity despite differing methodologies. When assessing the risk associated with different family members they found the results summarised in *Table 1.1*. The results of this study highlights that relative risk increases with increasing number of first degree relatives affected by the disease and younger age at diagnosis.

These results were replicated by a second re-analysis by the Collaborative Group on Hormonal Factors (2001), who looked at 54 studies in total(10). Initial analysis of the population across the studies highlighted that 12.9% of cases and 7.3% of controls had one or more affected first degree relative. This therefore demonstrates that a greater number of women with breast cancer also had an affected relative. The risk increased significantly with increasing numbers of first degree relatives affected: RR = 1.8, 2.93 and 3.9 for 1, 2 and 3 or more affected first degree relatives respectively(10). This risk further increased if the relative was diagnosed with early onset breast cancer. Once again these results

showed homogeneity across methodology, as regardless of how the study was carried out the results remained consistent, and when adjusted for various confounding factors.

Family Member	*RR (95% CI)*
Any relative	1.9 (1.7-2.0)
1st Degree Relative: All Ages <50 years ≥50 years	 2.1 (2.0-2.2) 2.3 (2.2-2.5) 1.8 (1.6-2.0)
Mother: All Ages <50 years ≥50 years	 2.0 (1.8-2.1) 2.0 (1.7-2.4) 1.7 (1.5-2.0)
Sister: All Ages <50 years ≥50 years	 2.3 (2.1-2.4) 2.7 (2.4-3.2) 2.0 (1.7-2.4)
Mother and Sister	3.6 (2.5-5.0)
2nd Degree Relative	1.5 (1.4-1.6)

Table 1.1 - A summary of the results of the meta-analysis performed by Pharoah et al (9), demonstrating the pooled estimated relative risk associated with each family member affected by cancer. These results also highlight that the younger that a relative is affected then the greater the risk.

The Nurses' Health Study was in agreement with the two above larger studies(11) and also highlighted that age had a large influence on risk. Those with a mother affected <40 years had a relative risk of 2.1 and if the mother was affected when she >70 years women had a far lower relative risk of 1.5.

Family history is key in predicting risk and a recent paper has suggested that in clinical practice, family history is a better predictive tool for breast cancer risk than the Gail model(12). This is reflected in the approach taken by the UK NICE guidelines, which emphasises the importance of family history when assessing a woman's risk of breast cancer and therefore identifying the most appropriate screening pathway(13). Family history is an established breast cancer risk factor which includes a large genetic component of the disease. Breast cancer genetics are very heterogeneous and there are a large number of genetic factors which can contribute to an individuals' risk of the disease.

3. Highly Penetrant Autosomal Dominant Breast Cancer Genes

As demonstrated by the epidemiological evidence above, breast cancer risk is increased when family members have been affected by the disease - especially if the cancer was early onset. It has been estimated that 10% of breast cancer cases are clustered within families(14). Due to this apparent inheritance of breast cancer, researchers began to look for the genetic basis of breast cancer in these families. To date they have found a number of genes which are linked to familial breast cancer; however the two genes which account for the highest proportion of inherited breast cancers are breast cancer 1, early onset (*BRCA1*) and breast cancer 2, early onset (*BRCA2*). Other genes have been identified to increase the risk of breast cancers about 2 fold; for example *CHEK2* and *ATM*(15). However there are also a number of lower penetrance genes which contribute to risks in these families and in sporadic breast cancer cases too.

Throughout this chapter the current literature on the genes involved in the inheritance of familial breast cancer shall be reviewed, to provide a short summary of what is currently understood about the higher penetrance genes and their encoded proteins.

3.1 BRCA1 and BRCA 2

BRCA1 and *BRCA2* where first identified in the 1990's (16–18) through linkage studies in large breast cancer families. *BRCA1* was initially identified by Hall et al, on chromosome 17, who looked at 23 families with 146 cases of early onset familial breast cancer(16). *BRCA2* was identified 4 years later on chromosome 13 by Wooster et al(17). Germ-line mutations in these two genes are thought to account for 15-20% of familial breast cancers(19), but there is still a great deal unknown about breast cancer genetics. *BRCA1* and *BRCA2* are responsible for autosomal dominant breast cancer inheritance, and both genes are about 80% penetrant by 70 years of age(20). It is also important to note that *BRCA1* and *2* both

also predispose to ovarian cancer, and increase the risk of prostatic, pancreatic, stomach cancer and melanoma(21).

In the Ashkenazi Jewish population there is a higher rate of both *BRCA1* and *BRCA2* mutations. A single mutation (185delAG) of *BRCA1* is responsible for 20% of early onset breast cancer in this population. A further 8% of early onset disease is due to a specific deletion in the *BRCA2* gene(22).

Women with sporadic cancers are also commonly found to have somatic mutations and altered expression of *BRCA1* and *BRCA2*(14), which is also common in ovarian cancer. Some women with germ-line mutations in these genes have also undergone a second somatic mutation, which causes the loss of wild type allele expression in the tumour(14,21). Once again there is ongoing research into the implications of these somatic mutations in both BRCA carriers and sporadic cases, but their significance in terms of risk or potential therapies is still unclear.

The proteins encoded by *BRCA1* and *BRCA2* can be thought of as caretakers, their function is to ensure that cellular stabilisation processes are working correctly. Loss in their function can lead to instability and mutations in gatekeeper genes which causes the loss of normal cellular controls(14). Both genes function within DNA damage repair pathways and are also commonly thought of as tumour suppressor genes(21).

They are involved in the recognition and repair of double strand DNA damage via homologous recombination(21). Double stranded breaks are more complex to repair due to the lack of complementary strand to use as a template, so choosing the correct repair pathway is critical(23). The process of homologous recombination takes place during the S and G_2 phase of the cell cycle, when the sister chromatids are available to use as a template(23). The protein encoded by *RAD51* is essential for this process and co-localises

with both BRCA1 and BRCA2 proteins. However the roles of BRCA1 and BRCA2 within this pathway differ. BRCA2 binds directly with RAD51 recombinase, and is thought to be involved in transporting RAD51 to damaged areas of DNA(23). Whereas BRCA1 is known to be needed for homologous recombination, but relatively little remains known about how or why it is needed(14). BRCA1 protein is also known to co-localise with RAD51 but not bind, however the significance of this is unknown(24).

Genes mutated in Fanconi's anaemia are also part of this repair pathway providing a link between it and the BRCA mutations(25). Fanconi's anaemia is an autosomal recessive disease with 13 different subtypes, which can be caused by mutations in 12 different genes(26). A biallelic mutation in the *BRCA2* gene can cause a subtype of Fanconi's anaemia called FA-D1, which is one of the few associated with childhood solid tumours. Other genes associated with familial breast cancer including *PALB2*, which shall be discussed later, are also involved in the inheritance of Fanconi's anaemia(26). It is clear that a common pathway is involved however the relevance of this is as yet unknown. However it may provide avenues of research in terms of further elucidating breast cancer genetics, which has been a difficult field of research due to its heterogeneity.

Double strand repair and chromosomal stability are linked, as without correct repair the chromosome losses stability(14). It is therefore logical that BRCA1 and BRCA2 proteins are thought to both play a role in the stability of the chromosome throughout the cell cycle(27). Loss of this stability is hypothesised to cause translocations, large duplications and fusions during the normal process of cell division(27). These are all processes which have been linked to carcinogenesis.

One of the BRCA1 proteins binding partners is BARD1 (BRCA1-associated RING domain protein) together they form a heterodimer, which functions as an ubiquitin ligase. This complex plays a role in DNA repair, centrosome regulation and transcription. The BRCA1

protein binds to a gamma tubulin which is part of the centrosome and its ubiquitin ligase activity is thought to prevent the centrosome from being overactive(28).

More recently BRCA1 has been linked to a role in autophagy, which is the process by which organelles and proteins are degraded. It is still unknown how the BRCA1 protein regulates autophagy, but when its protein expression is decreased autophagy is promoted(29). This promotion of autophagy has been shown to have a function in malignant progression and autophagy markers are high in breast cancer(29).

An additional essential role which both gene products have is in the regulation of the cell cycle, but once again the two differ in the exact nature of this role. BRCA1 protein is a regulator of the G_2-M phase of the cycle(23), which is the transition from chromosomal duplication to mitosis. Whereas BRCA2 protein relates more to the progression through mitosis and the M phase check point(23). Their involvement in the cell cycle once again relates to chromosomal stability and genomic integrity of the cellular contents, so if this process goes wrong it is likely there will be an increased instability and an increase in genetic mutations.

The functions of BRCA1 and BRCA2 are complex and there is still much to be learned which could influence clinical management of patients in the future. Some important questions which remain to be answered include: why do these ubiquitously expressed proteins promote breast and ovarian cancer? And are these high penetrance genes influenced by the low penetrance polymorphisms(19,30)?

3.2 Other Potential Candidate Genes

BRCA1 and *BRCA2* were the first identified and best studied familial breast cancer genes, however they only account for 15-20% of familial risk(14,19). This therefore means there is a great deal still unknown about the inheritance of familial breast cancer. This has led to a large amount of genetic analyses to find other genes which are associated with breast

cancer risk in these high risk families; however due to the heterogeneous nature of breast cancer genetics identifying other high risk genes like *BRCA1* and *2* has been extremely difficult. Despite these difficulties over the last two decades there have been a number of genes shown to increase breast cancer risk – these include *PTEN, TP53, PALB2, ATM* and *CHEK2*.

The *PTEN* gene encodes a phosphatase and tensin homolog protein which acts as a tumour suppressor. Mutations in this gene cause the autosomal dominant cancer predisposition syndrome Cowdens disease. This predisposes affected individuals to thyroid, breast and skin cancer(31,32). There is increasing evidence that germ-line mutations in *PTEN* may also predispose to non-Cowdens disease related high risk familial breast cancer(31). This is a high risk gene which has a differing effect depending on the specific mutation, but which is a rare breast cancer predisposition gene.

A second high risk breast cancer risk gene is *TP53* which encodes the tumour suppressor gene p53, which is also associated with a rare cancer predisposition syndrome - Li-Fraumeni Syndrome(2). p53 is involved in cell transformation, cell cycle check-points, DNA repair and mediated apoptosis(33). Various studies have associated a number of germ-line mutations with familial breast cancer risk and one paper estimated that 1% of inherited breast cancers have a germ-line *TP53* mutation(33). Additionally mutations in this gene seem to be an early event in sporadic breast tumourigenesis, and p53 expression is altered in 20-40% of sporadic breast cancer(34). *TP53* is a gene that is central to the predisposition of disease and is also involved in the progression of sporadic disease. However as these mutations are rare *TP53* accounts for a small proportion of high risk disease.

The second set of genes involved in familial breast cancer are all associated with a moderate risk of breast cancer, causing approximately a 2 fold increase in risk(2). *PALB2* (partner and localiser of BRCA2) is thought to infer a higher risk, up to an 8-9 fold increase

in risk, than the other genes discussed in this section(35). It encodes a protein which co-localises with BRCA2 and may function as a tumour suppressor(36). It is thought to function in the same DNA repair pathway as BRCA2, a hypothesis which is supported by the fact that *BRCA2* and *PALB2* mutations have the similar breast cancer phenotypes(2). Additionally both *BRCA2* and *PALB2* are both mutated in different subtypes of the autosomal recessive condition Fanconi's anaemia. The subtypes they are associated with FA-D1 and FA-N, respectively, are also associated with a risk of solid tumours in childhood and function downstream of the FANCD2 complex(26).

PALB2 mutations have been associated with an increase in breast cancer risk in small but substantial number of breast cancer families(37); one study has estimated that the prevalence was 1% in an a group of Australian women with triple negative breast cancers(36). Mono-allelic mutations in *PALB2* have been found to confer a 2 fold increase in risk in breast cancer families(26,36). There has also been indications that *PALB2* mutations predispose to an early onset breast cancer opposed to a late onset phenotype; the relative risk of breast cancer <50 years old was 3.0 and 1.9 for over 50's(26). These results have been confirmed in a group of 362 people from 154 families; which found an 8-9 fold increase in risk in those younger than 40 years old, 6-8 fold increase in risk in those 40 to 60 years old and 5 fold increase in risk in those over 60 years old(35). There is clear evidence that mutations in *PALB2* has an impact on familial breast cancer, but it is still unknown if mutations in this gene alone is enough to cause multiple breast cancer cases in one family.

CHEK2 (Checkpoint kinase 2) encodes a cell cycle regulator, which has also been associated with a two fold increase in breast cancer risk(38,39). This gene was first associated with breast cancer in a linkage study of a large breast cancer family(2), however mutations in *CHEK2* are rare and only found in a small proportion of breast cancer families(38).

Mutations in this gene are thought to have a 4-5% prevalence in familial breast cancer cases, and the prevalence is thought to increase as the number of cases within the family increases(40–42).

The protein encoded by CHEK2 is, as mentioned above, a check point regulator which phosphorylates the tumour suppressor p53 and BRCA1 both of which have been previously associated with familial breast cancer. Phosphorylation of these proteins modulates the function of p53 to arrest the cell cycle in response to DNA damage(43). Once again there is some uncertainty if a mutation in this gene alone could account for the clustering in families or if other mutations must also co-exist. Additionally as with TP53, CHEK2 is also associated with the cancer predisposition syndrome Li—Fraumeni syndrome(43).

ATM (Ataxia Telangiectasia Mutated) encodes a protein required for the activation of CHEK2 protein and other check point proteins in response to ionising radiation(41). This gene was initially chosen for investigation because it had been noted that suffers of the rare neurological genetic condition Ataxia Telangiectasia and carriers had higher rates of breast cancer(44,45).

Heterozygous carriers of certain *ATM* mutations are thought to present with a 2-4 fold increase in breast cancer risk, additionally there is no difference in risk between women under and over 50 years old(46,47). This is a higher increase in risk than *CHEK2*, it is thought that the specific mutation in the *ATM* gene may influence the level of risk conferred by the mutated allele. For example one study found two particular mutations which were 60% penetrant by 70years old, which is equivalent to a 15 fold increase in risk(48) this is far greater than the 2-4 fold risk increase predicted by other studies.

All of the genes that have been discussed play a role in breast cancer genetics, and confer a moderate to high risk of developing breast cancer. It is interesting to note that these five

genes converge in a common DNA repair pathway with BRCA1 and BRCA2. However together *BRCA1, BRCA2, PTEN, TP53, PALB2, CHEK2* and *ATM* probably only account for 20-25% of inherited breast cancer risk, therefore there are still a number of genes yet to be identified. These unidentified genes are likely to be a mixture of both highly penetrant genes and low penetrance polymorphisms. Additionally the search for more candidate risk genes could potentially be centred on genes which also sit within this common DNA repair pathway.

4. Low Penetrance Polymorphisms and Breast Cancer Risk

Due to the recent emergence of genome wide association studies (GWAS) there have been a number of studies which have identified low penetrance polymorphisms associated with breast cancer (*Table 1.2)*. These are not directly causative of breast cancer but do increase an individual's risk of developing the disease. For example women with 14 of the 18 the risk alleles have a 6 fold increased risk of developing breast cancer compared to a woman with none of the risk alleles(49). This polygenic model of breast cancer risk suggests that multiple low risk variants have an additive effect, which may eventually reach a threshold of disease when added with environmental and lifestyle risk factors(50). These single nucleotide polymorphisms (SNPs) are believed to be responsible for 8% of excess familial risk(51).

There is currently little known about how these small genetic changes predispose women to the development of breast cancer. It is thought that these changes perhaps subtly alter a proteins function or alter interactions within specific pathways(52). Below is a summary of the loci in which breast cancer related polymorphisms have been identified (*Table 1.2*). For each of these loci there have been candidate genes identified, which are thought to be the most likely gene responsible for the effect on risk. However there is also a possibility that these genes may not be the reason for the alteration in risk seen, there may be other

genetics elements influencing breast cancer risk. For example within these regions of DNA there may be regulatory regions such as an enhancer or silencer which can be several hundred kilobase pairs from the promoter region. Or additionally there are locus control regions which are groups of control elements that can regulate the transcription of genes involved in tissue specific expression. These are just some of the transcriptional control elements which flank genes and could be alternatives to the genes identified as being involved in breast cancer risk(53).

Of these candidate genes there is relatively little known about why they may be associated with breast cancer. Therefore each of these polymorphisms which lie within a gene, or with a candidate gene, shall be discussed in turn with regards to their function and possible association within breast cancer.

Gene	Locus	SNP Identifiers	Per Allele OR	Source
FGFR2	10q26	rs2981582	1.26	Easton et al 2007(54)
TNRC9 (TOX3)	16q12	rs12443621	1.11	Easton et al 2007 (54)
	5p12	rs4415084	1.19	Stacey et al 2008(55)
NOTCH2	1p11	rs11249433	1.16	Thomas et al 2009(56)
ZNF365	10q21	rs10995190	0.86	Turnbull et al 2010 (57)
RAD51L	14q24	rs999737	0.94	Thomas et al 2009(56)
ESR1	6q25	rs2046210	1.36	Zheng et al 2009 (58)
	11q13	rs614367	1.15	Turnbull et al 2010 (57)
CASP8	2q33	rs1045485	0.89	Cox et al 2007 (59)
	2q35	rs13387042	1.12	Milne et al 2009 (60)
NEK10, SLC4A7	3p24	rs4973768	1.11	Ahmed et al 2009(61)
MAP3K1	5q11	rs889312	1.13	Easton et al 2007(54)
CDKN2A/B	9p21	rs1011970	1.07	Turnbull et al 2010(57)
	8q24	rs132281615	1.08	Easton et al 2007(54)
LSP1	11p15	rs3817198	1.07	Easton et al 2007(54)
	10q22	rs704010	1.07	Turnbull et al 2010(57)
	10p15	rs2380205	0.94	Turnbull et al 2010(57)
COX11	17q	rs6504950	0.95	Ahmed et al 2009 (61)

Table1.2 Summary of the low penetrance polymorphisms involved in breast cancer risk

4.1 Fibroblast Growth Factor Receptor 2

Fibroblast Growth Factor Receptor 2 (*FGFR2* – located on 10q26) is the most strongly associated susceptibility locus for breast cancer(62); there is a 2 fold increase in risk in women homozygous for one particular single nucleotide polymorphism (SNP) in

comparison to those homozygous for the wild type allele(63). To date there have been 5 different SNPs in *FGFR2* intron 2 associated with an increased risk of breast cancer(54,64,65).

FGFR2 is part of the fibroblast growth factor family, which includes 22 fibroblast growth factors and 4 tyrosine kinase receptors. These receptors, including FGFR2, are activated by the binding of a fibroblast growth factor (FGF) and heparin/heparin sulphate proteogylcan (HSPG) to the receptor which causes dimerization and autophosphorylation, followed by the activation of an appropriate signalling cascade. These signalling cascades include the ras-MAPK pathway and the IP3 or DAG pathways(66).

The FGF family of signalling factors are involved in embryonic development (cell proliferation, differentiation and migration), adult tissue homeostasis (tissue repair, wound healing and angiogenesis)(66) and mammary gland development(7).

FGFR2 itself exists in two differing isoforms, b and c, which differ in their alternative splicing of exons 9 and 10(67). These differing isoforms have different binding partners and expression patterns – FGFR2 b is expressed in endothelial cells and FGFR2 c is found in mesenchymal cells(66).

Alterations in the *FGFR2* gene have been associated with a number of cancers including breast cancer, gastric cancer, lung and ovarian cancer(67). It has also been found to be overexpressed in 10-15% of breast tumours(7). Several studies have found that the rs2981578 SNP in FGFR2 intron 2 is associated with oestrogen receptor positive (ER) tumours(7,64) and there is also a small body of evidence indicating that this SNP is also associated with PR positive tumours(68). This is suggestive that specific genetic risk factors may predispose to certain tumour characteristics or breast cancer subtypes.

There have also been some studies into interactions between *FGFR2* polymorphisms and other modifiable risk factors such as smoking or alcohol consumption. One example of such a study was carried out by Marian et al (2011)(64), they investigated the relationships between the *FGFR2* gene and smoking, alcohol consumption and obesity in breast cancer. No associations were found between *FGFR2* and obesity or alcohol consumption, but there was a correlation between *FGFR2* and smoking. This study found a 2 fold increase in risk in smokers with the *FGFR2* polymorphism in comparison to non-smokers. However the authors do state that there may have been a short fall in their statistical power due to a small number of non-smoking controls. Having said this it is an interesting finding that would be exciting to pursue further as it may elucidate the mechanisms by which *FGFR2* confers risk.

There is still little understood about how the *FGFR2* polymorphisms are involved in breast cancer pathogenesis, however there are some indications it could be related to differential levels of expression and hormonal pathways(7). This is supported by the association with ER positive tumours and the elevated levels of FGFR2 expression seen in 10-15% of breast tumours. Intron 2 has also been found to be highly conserved in mammals and is thought to have a number of transcription factor binding sites, some of which lie relatively close to the SNPs identified by GWAS(54). Alterations at these transcription factor binding sites may represent the mechanism by which expression is altered in women with particular SNPs.

4.2 Trinucleotide Repeat Containing Gene 9

Trinucleotide repeat containing gene 9 (*TNRC9*) is otherwise known as *TOX3* was identified by Easton et al (2007)(54), which has since been replicated by other groups in different populations(7,69,70). It is hypothesised to be a transcription factor due to the presence of a high mobility group (HMG)(54).

When this loci was examined in a Dutch cohort for clinical correlations there was indications that it may be associated with younger age of diagnosis(71). Studies have also linked *TNRC9* with 3-4% increase breast density(72), which is an established breast cancer risk factor as discussed later. Several other polymorphisms have also been marginally associated with an increase in breast density including *LSP1*, *MAP3K1*(73) and *ZNF365*(74). *LSP1* and *MAP3K1* have been estimated to account for 1.5% variation in breast density(73) and *ZNF365* is thought to account for 0.5% of variation(74).

However despite these associations with clinical factors there is very little understood about what the protein encoded by *TNRC9* actually does. In the brain TNRC9 protein has been shown to be involved in calcium dependent transcription(75). This transcription has been shown to be essential for cell survival in neurones and *TNRC9* is thought to encode a neuronal survival factor(76). It has also been shown to be expressed in high levels in the central nervous system but is not normally expressed in breast tissue(76). It is still unclear what the implications of these finding have on the role of *TNRC9*'s in breast cancer risk. However it will be interesting to see if there is TNRC9 expression in breast tumours and if could potentially play a role in tumour cell survival.

4.3 5p12

This locus was identified by Stacey et al in 2008, and there is only one gene in this interval which is a candidate for altering breast cancer risk. This is programmed cell death protein 9 (*PDCD9* aka *MRP530*) which is a small mitochondrial subunit thought to be involved in pro-apoptotic events(55,77). The authors chose to look at this gene in particular as it had be identified in several previous GWAS studies but had never reached statistical significance. PDCD9 is not normally expressed in luminal epithelial cells, but has been shown to be up regulated in infiltrating ductal breast cancers(55).

However it is still unknown if this polymorphism relates to *PDCD9,* and if the risk is associated with this gene the mechanism underlying this relationship. It may relate to its pro-apoptotic effects, and the polymorphism may make cells more resistant to apoptotic processes.

4.4 NOTCH 2

NOTCH 2 has been associated with breast cancer in several GWAS studies(56,78); one of these studies found that *NOTCH2* polymorphisms where associated with ER positive tumours(78). NOTCH2 is one of a family of transmembrane receptors which interacts with the membrane bound ligands Jagged 1, Jagged 2 and Delta 1. Upon binding there is a cleavage event releasing a NOTCH intracellular domain which enters the nucleus to influence the expression of target genes(78). This NOTCH signalling plays a key role in neurodevelopment and cell fate decisions.

NOTCH 1 and 4 have already been shown to be overexpressed in some breast cancers(79). So it is conceivable that there is a role for *NOTCH 2* polymorphisms in the pathogenesis of some breast cancers.

4.5 Zinc Finger Protein 365

Zinc Finger Protein 365 (*ZNF365*) was first discovered to be associated with breast cancer risk in 2010 by Turnbull et al(57). This particular zinc finger protein is also known as *Su48*, which has also been identified as a centrosomal protein. This 407 amino acid polypeptide co-localises with the centrosome throughout the cell cycle. In a series of experiments in which the expression of ZNF365 was altered, it was found that it played an essential role in mitosis(80). This role in mitosis may offer a mechanism by which a polymorphism in this gene could cause an alteration in the risk of breast cancer.

On another note, an alternative polymorphism in intron 4 of *ZNF365* has been shown to be associated with a decreased mammographic density. This could be thought of as a protective polymorphism as an increased density increases breast cancer risk - >75% dense tissue has a 4 to 5 fold increase in breast cancer risk(74). Further research would be needed to identify if different polymorphisms in this gene could increase breast density. There is clearly a complex relationship between breast cancer risk and *ZNF365*, as different polymorphisms can both infer an increased or decreased risk.

4.6 RAD51 Homolog B

RAD51 Homolog B (*RAD51L1*) was identified by Thomas et al by a 3 stage GWAS study and is a member of the RAD51 protein family involved in DNA repair(56). It encodes one of the 5 paralogs of *RAD51*(51), which as mentioned above is involved in the repair of double strand breakages via homologous recombination. RAD51L1 is not thought to directly interact with the damaged DNA itself but instead to play a role in junction processing during the process of homologous recombination(51). In addition there has been a suggestion that its protein may play a role in cell cycle control and apoptosis as it has been shown to interact with p53(51).

It has also been shown that like some of the other low penetrance polymorphisms there has been an association between polymorphisms in *RAD51L1* and mammographic density, which is associated with risk(81). However none of the SNPs in this gene have been associated with a specific subtype of breast cancer, it appears be associated with all tumour types including triple negative(82).

This gene, like many other genes implicated in breast cancer risk, is involved in DNA repair pathways. This highlights the importance of loss of genomic stability in the progression of breast carcinogenesis(51).

4.7 Oestrogen Receptor 1

Oestrogen Receptor 1 is the most likely candidate gene identified in the locus found to be associated with breast cancer by Zheng et al(58). The SNP lies 180Kbp upstream of the translation start site and 26kbp of the first untranslated exon. Other genes in this area included *PLEKHGI, MTHFDIL, AKAPI2, ZBTB2, RMNDI, C6orf211, C6orf98* and *SYNE1*(58). However due to the oestrogen receptors established role in breast cancer pathogenesis *ESR1* has been pursued as the most likely gene to be involved in conferring an increased risk of breast cancer(58,83). Other studies have reproduced similar results in the same locus in different study populations, further supporting a role for this region in breast cancer risk(84).

The *ESR1* gene codes for oestrogen receptor (ER) alpha which can be elevated in both pre-malignant and malignant tumours, and is both a predictive and prognostic factor in terms of clinical management(83). Upon activation the ER can regulate the transcription of target genes, which regulate the growth and differentiation of normal breast tissue(85). There has also been some evidence that the *ESR1* gene is amplified in a proportion of breast cancers(86), however the quoted values vary widely from 20%(87) to 5.9%(85,88). There have also been indications that clusters of *ESR1* accumulate in the nucleus in some tumours, which is not seen in normal breast tissue. However the reason for this or its implications are as yet unknown(85,86). It is also still unknown if there is any connection between this gene amplification and the polymorphism in the region of the *ESR1* gene. The exact mechanism by which *ESR1* increases breast cancer risk, and indeed if the SNPs in this region actually relates to *ESR1* opposed to another candidate gene remains unknown.

4.8 Caspase 8

Caspase 8 (*CASP8*) was identified as a gene in which polymorphisms have an impact on breast cancer risk(59) in a case control analysis by the breast cancer association

consortium. One polymorphism was recognised to be protective against breast cancer - it was a single amino acid change of aspartic acid to histidine(59). Additionally there have been *CASP8* polymorphisms associated with increased risk of developing breast cancer(89).

CASP8 encodes one of the initiating factors for apoptosis; it is a pro-enzyme which means that is activated by cleavage by a caspase activating complex. In the case of caspase 8 the activating factor is DISC, and together these are both part of the extrinsic pathway of apoptosis. The extrinsic pathway relies on a death receptor to remove unwanted or damaged cells(90). There have also been suggestions that caspase 8 has other moonlighting functions out-with its role in apoptosis; these functions include roles in cell migration and cell matrix adhesion(91).

There has been some work done to investigate how CASP8 variants may affect apoptotic processes and thus have some influence over an individual's risk. A study which looked at the effect of the polymorphism on caspase-8 activity in peripheral lymphocytes found that different alleles had different levels of activity and apoptosis. It is therefore conceivable that these alleles alter breast cancer risk by altering caspase-8 activity and its apoptotic actions. However further work on the subject is needed to confirm these results(89).

4.9 NEK10 and SCL4A7

At the locus 3p24 which was associated with breast cancer risk by Ahmed et al there were two plausible candidate genes suggested by the authors(61). These were Never in mitosis related kinase 10 (*NEK10*) and solute carrier family 4, sodium bicarbonate co-transporter member 7 (*SLC4A7*) each of these shall be discussed in turn focusing on their mechanism of action and potential involvement in breast cancer risk.

The *NEK10* gene is part of a family of genes which control cell cycle but it is still unknown what its specific role is(61). It encodes a serine/threonine protein kinase which has

recently been suggested to play a role in checkpoint control, more specifically the G2/M checkpoint. However this work was all carried out in cell lines and further research will be needed to confirm its specific role(92). *NEK10* has also been implicated in other cancers including lung cancer(92). Both its proposed function and a previous association with human cancer confirm this as a plausible gene to be involved in breast cancer risk.

SLC4A7 on the other hand, encodes a bicarbonate co-transporter (61), which has been shown to be down regulated in 64% of tumours in a small case control sample(93). This co-transporter facilitates the movement of bicarbonate which controls intracellular and extracellular pH. In tumour cells the intracellular pH tends to be more alkaline and the microenvironment more acidic (by approximately 0.2 pH)(93). This relationship tends to be more pronounced in more aggressive cancers(94). The difference in pH is thought to be due to a number of transporters including the one encoded by *SLC4A7*(94). Currently the role of this transporter in controlling the pH of cells and the microenvironment in tumourigenesis is unknown, but this provides a plausible mechanism by which changes in this gene could increase breast cancer risk.

It is still unclear which of these genes is responsible for the increased risk associated with 3p24 locus identified by Ahmed and replicated by other groups(61,70,95). However it can be seen that both have plausible mechanisms of action in terms of increasing breast cancer risk, but more research is needed to disentangle which gene is involved.

4.10 Mitogen-activated Protein Kinase Kinase Kinase 1

Easton et al(96) identified the SNP rs889312 in the *MAP3K1* gene was associated with breast cancer in 2007. Since there has been some evidence that *MAP3K1* polymorphisms are associated with the oestrogen and progesterone receptor positive tumours(71).

Mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) is located on chromosome 5q11.2 and encodes serine/threonine kinase. It is part of the MAP kinase signalling

pathway that includes Ras, Raf, MEK and ERK, and is critical in cellular regulation(97). Its mechanism of activation is by the process of autophosphorylation, but when phosphorylating other proteins MAP3K1 requires a magnesium co-factor(98).

MAP3K1 is involved in the transcriptional control of some key genes which have been linked to tumourigenesis: c-Myc, c-Elk1, c-Jun and c-Fos. This transcriptional control relates to the ability of MAP3K1 to activate MAPK2 via the process of phosphorylation. Active MAPK2 then phosphorylates MAPK/ERK which trigger downstream signalling cascades to control transcription(99). This signalling pathway has also been linked with HER2 receptor activity, another receptor which is up regulated in some breast cancers(99). The MAP3K1 pathway also converges with the FGFR2 signalling pathways, so having both polymorphisms may hypothetically have an effect on breast cancer risk. Once again further work as to how this gene confers risk and its prevalence in the population is required to fully understand the impact of this polymorphism.

4.11 Cyclin-Dependent Kinase Inhibitor 2 A/B

Polymorphisms associated with breast cancer risk in cylcin-dependent kinase inhibitor 2 A/B (*CDKN2A/B*) were discovered in 2010 by Turnbull et al(57). There are 2 differing isoforms which is due to transcription of the first exon in one and not the other, hence the A/B. The gene itself encodes a cell cycle regulator, which has previously been identified as a potential oncogene or tumour suppressor gene(100). Very little is known about this gene in relation to breast cancer, but there are indications that like other breast cancer risk genes it is involved in cell cycle control; this once again highlights the convergence of these breast cancer risk genes within the same cellular processes and signalling pathways.

4.12 Lymphocyte Specific Protein 1

Lymphocyte Specific Protein 1 (*LSP1*) encodes an intracellular F actin binding cytoskeleton protein which was associated with breast cancer risk by Easton et al(96). This finding has been replicated in a large meta-analysis, which aimed to come to a conclusive decision on the relationship between this allele and breast cancer risk(101).

The encoded F actin binding protein is expressed in lymphocytes and endothelial cells(50). It may be involved in neutrophil motility, cell adhesion and trans-endothelial migration(101). This function is thought to be guided by MAP kinase pathway and its normal function it thought to allow cells to travel to sites of injury.

This gene has already been previously associated other malignancies including Hodgkins lymphoma and other haematological malignancies(50,102). However its role in breast cancer is somewhat less clear, and further work will be needed to understand how polymorphisms in *LSP1* could increase breast cancer risk.

4.13 Cytochrome C Oxidase Assembly Protein 11

Ahmed et al identified 17q23.2 as increasing the risk of breast cancer, the SNP lies within an intron of syntaxin binding protein 4. *STXBP4* is a novel insulin regulated syntaxin 4-binding protein which directly interacts with p63 to stabilise ΔNp63 and is involved in glucose and GLUT4 vesicular transport(103).

However Cytochrome C oxidase assembly protein (*COX*) 11 lies within close proximity to the SNP. *COX 11* encodes a protein which is responsible for the assembly of cytochrome c oxidase a crucial part of the respiratory complex(104). Expression of COX11 was found to be increased in tumours and therefore has been taken forward as the candidate gene in this region, however the relationship between breast cancer and this polymorphism remains unclear(61).

There are numerous low penetrance polymorphisms which have been identified as playing a role in breast cancer susceptibility. Polymorphisms which both increase and decrease risk have been identified within the same gene. The genes implicated are across a wide variety of processes but there are one or two pathways which seem to be crucial. These include DNA repair pathways, in particular homologous repair pathways, which is implicated by both high and low risk mutations. The broad categories of processes which have been implicated by these SNPs are DNA repair, cell cycle, microenvironment, apoptosis, transcription and oestrogen signalling. This begins to illustrate that the breast cancer polymorphisms tend to cluster in common broad processes, which are potentially involved in breast cancer pathogenesis. This may begin to explain the additive effect having multiple polymorphisms has on risk.

5. Summary

Breast cancer genetics is extremely heterogeneous and still very little remains known despite the two decades of research since the discovery of BRCA1. As previously stated a number of higher penetrance mutations have been identified in a number of breast cancer families, these include *BRCA1, BRCA2, PTEN, TP53, CHEK2, ATM* and *PALB2*. There are also a variety of low risk polymorphisms which increase the risk of disease or susceptibility but are not causative. These SNPs are spread across a variety of pathways and in general confer a small risk which will reach a threshold of disease when other risk factors are taken into account such as parity and lifestyle factors.

There is also a tendency for the proteins encoded by these high and low risk genes to be involved in several common pathways including DNA repair, cell cycle control, and control of the microenvironment as demonstrated by *Figure 1.1*. In addition to the convergence in functionality of these genes there are also indications that there may be interactions between these genes and other known risk factors. For example a number of the low

penetrance polymorphisms have been associated with breast density (*LSP1, TNRC9, MAP3K1* and *ZNF365)*, a known risk factor which is covered in the following chapter. The interactions between the genetic and environmental risk factors shall also be discussed in a later chapter. This review of the genetic factors associated with breast cancer highlights two things: first that there are a vast number of diverse genes involved in the risk and pathogenesis of this disease. Secondly that there are certain cellular processes and pathways which are common to these risk genes and interactions may exist between the genes themselves.

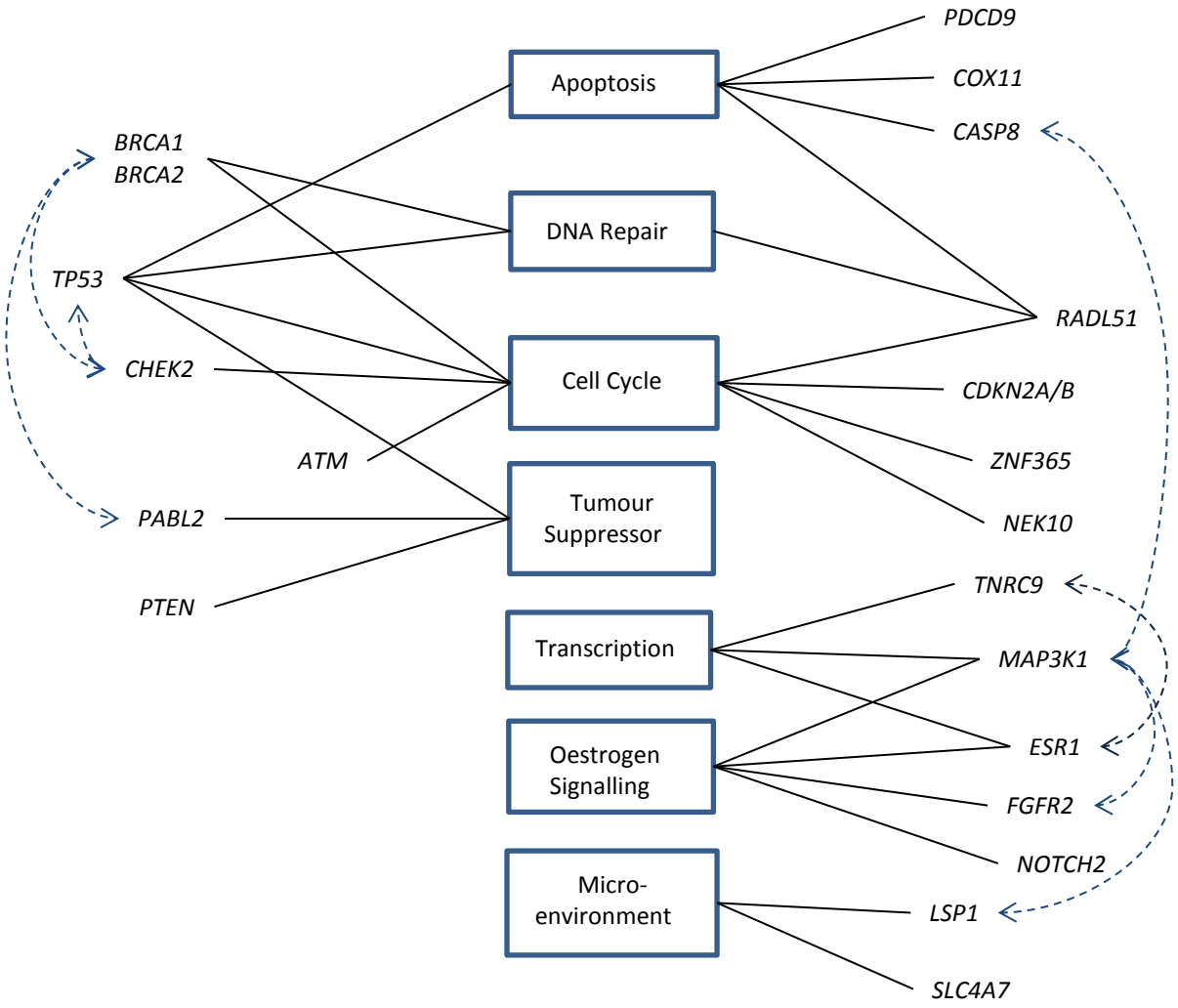


Figure 1.1 A summary of the genes which have been identified as modifying breast cancer susceptibility and the broad biological processes which they are involved in. This demonstrates that these genes are clustered within a few main cellular processes, which also that the proteins encoded by these genes interact with each other. The blue double headed arrows indicate interactions between the genes themselves.

Reproductive Hormonal and Environmental Factors Which Alter Breast Cancer Risk

1. Introduction

The highest incidences of breast cancer worldwide are found in Western Europe and other developed countries, and the lowest incidence of the disease is in the Asian Subcontinent(3). This differing incidence could be explained by a multitude of genetic and environmental factors. However evidence from migration studies suggests that it is the influence of environmental factors which best explains this differing breast cancer incidence worldwide. These studies show that the offspring of women from areas with a low incidence of breast cancer who have migrated to the USA, will have breast cancer rates the same as the US averages within a couple of generations(105). This indicates that the environment has an influence on breast cancer risk in addition to the genetic risks discussed previously.

Identification of these environmental risk factors contributing to breast cancer began in the 1970's. These epidemiological studies have now identified a variety of risk factors which have a generally accepted association with breast cancer risk. Some of the strongest associations have been between breast cancer and reproductive history and hormonal preparations. Early age at menarche, late age at first full term pregnancy (FFTP) and menopause (4-9) have all been linked to an increased risk of developing breast cancer. Other factors such as breastfeeding and multiparity have been identified as being protective against the disease(112–115). Prescribed hormonal preparations such as the oral contraceptive pill (OCP) and hormone replacement therapy (HRT) have also been associated with an increased risk of developing breast cancer(110,116–123).

Some potentially modifiable lifestyle factors and other environmental factors have also been investigated to determine their relationship with breast cancer risk; these include body mass index (BMI)(124–128), diet(129–138), smoking(139–143), alcohol consumption(139,144–147), ionising radiation exposure(148–151), ethnicity(119,152–154), socioeconomic status(155–157), mammographic density(158–164) and environmental exposure(165–170) to chemicals at work or in the home. Of these factors age, BMI, radiation exposure, mammographic density and alcohol consumption have all been studied extensively and are generally accepted as being involved in breast cancer risk.

To provide a comprehensive evaluation of environmental factors and breast cancer risk the evidence for the associations between a number of environmental and reproductive factors with breast cancer will be evaluated, including a brief description of the biological mechanisms believed to underlie this relationship.

The literature search in this case shall be a journalistic review of the vast epidemiological literature on the subject. The focus of the review shall be on larger meta-analyses, pooled analyses, systematic reviews, and larger cohort or case control based studies. Suitable articles were identified via Medline searches using breast neoplasms – aetiology/epidemiology, with an appropriate accompanying MeSH search term (see Appendix 2). The literature search was further expanded by exploring relevant papers within the references of the papers identified by the original Medline search.

2. Reproductive and Hormonal Factors

Ramizzini was the first to observe the link between a woman's reproductive history and risk of breast cancer in the 1700's. He observed that the rate of breast malignancy was greater in group of Italian nuns in comparison to the general population(105). This has continued be a popular subject of epidemiological study which has continued in recent years, and the consensus of this work is summarised below.

2.1 Age at Menarche and Menopause

Early age of menarche and late age at menopause have generally been accepted as increasing an individual's risk of developing breast cancer. Pike's theory of breast tissue ageing may help understand why these two reproductive events could influence the risk of developing breast cancer(171). It states that breast tissue begins the process of "ageing" at

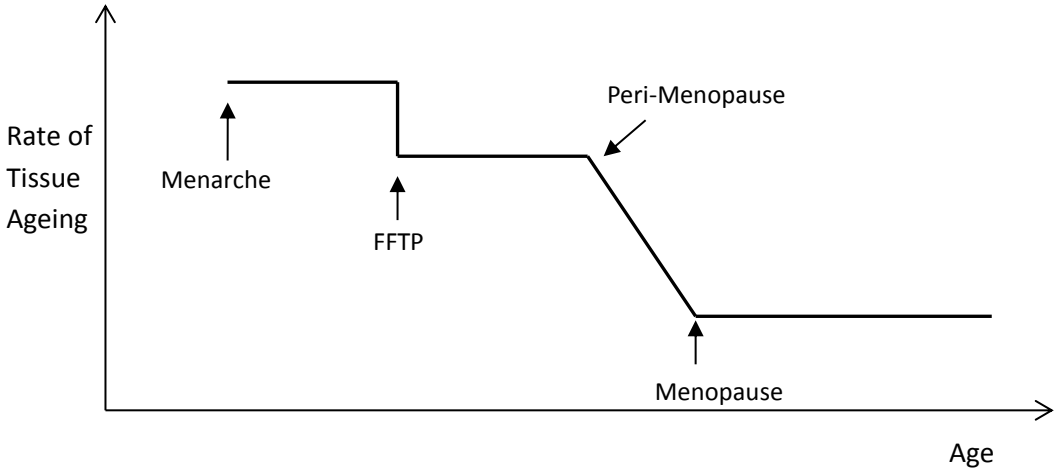


Figure 2.1: Simplified diagram demonstrating Pike's hypothesised pathway of breast tissue ageing. This process is influenced by hormone levels and events in a woman's reproductive life. Increased tissue age is thought to relate to an increased number of potential neoplastic changes which could happen within the cells. Adapted from Pike et al (1983) [65].

menarche; "ageing" is defined as proliferation, and differentiation accompanied by the potential to accumulate genetic mutations. This process is influenced by the levels of circulating reproductive hormones and continues until menopause. The rate of the ageing process varies with time and reproductive events – the highest rate is between menarche and FFTP, and then drops to a lower constant rate. During the peri-menopause this rate begins to decline again until menopause is reached (*Figure 2.1*).

Pikes theory of breast tissue ageing is in alignment with the developmental process of breast tissue, which occurs between menarche and FFTP, and is also influenced by levels of circulating sex hormones. After the first full-term pregnancy breast epithelium is fully differentiated, and at this stage is thought to be more resistant to carcinogens and the hormonal milieu of the breast(167). These endogenous hormones include oestrogens and

progesterones; both of which initiate proliferative and developmental activity within breast epithelial cells and are associated with breast cancer pathology(172). Menarche and menopause mark the beginning and end of ovarian activity and a woman's reproductive lifespan(173). Therefore if ovarian activity begins earlier and ends later breast tissues are exposed to these endogenous steroid hormones for longer period of time(111).

The association between early menarche and risk has been identified by various studies. One large meta-analysis by the Collaborative Group on Hormonal Factors in breast cancer (2012) looked at 117 studies from 35 different countries, which only included women who had never taken HRT. It found that there was a relative risk of (RR) = 1.05 (CI 95% - 1.044 – 1.057; p <0.0001) for every year younger than 13 years old a woman was at menarche (*Figure 2.2*). However in postmenopausal women it was found that an increased current body mass index (BMI) removed the increased risk associated with menarche and menopause(173). It is unknown why a high BMI attenuates this increased risk but it may relate to the mechanism by which a high BMI increases breast cancer risk in postmenopausal women, which is discussed in more detail later.

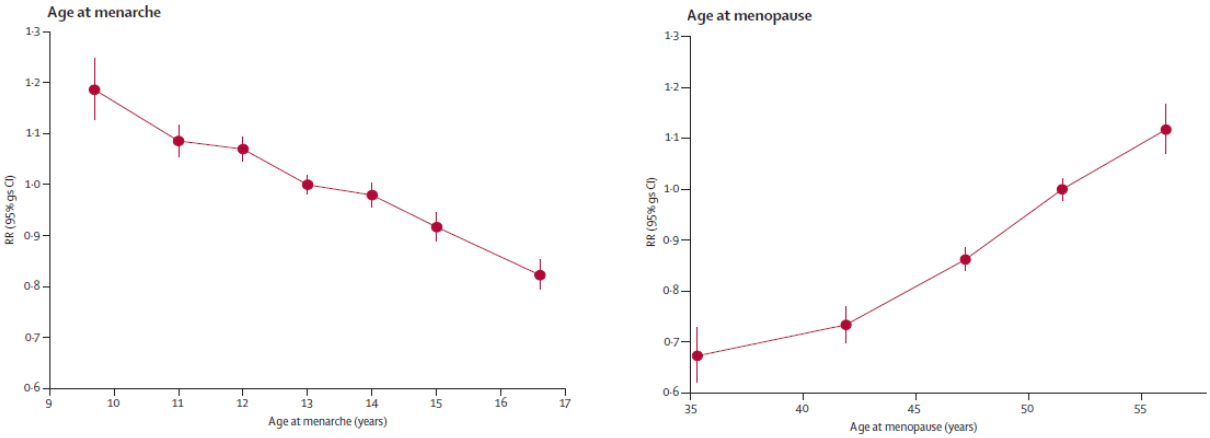


Figure 2.2 - Relative risks of age at menarche and menopause as estimated by a large collaborative reanalysis, which illustrates that with decreasing age at menarche and increasing age at menopause there is an increased breast cancer risk. Adapted from The Collaborative Group on Hormonal Factors (173).

As part of the Women's care study Li et al (2008), failed to find any association between age at menarche and breast cancer(108). Instead they identified a higher breast cancer risk in women with a larger age gap between age at menarche and FFTP in both pre- and postmenopausal women. For example in pre-menopausal white women who had a ≥16 year gap between the two events had a 1.5 fold increased risk, when compared with those who had a gap of <5 years between menarche and FFTP. These results must be interpreted with caution as there were small numbers of participants when they were divided into subgroups for analysis. Additionally as with all case-control studies issues such recall and selection bias must be considered. These results do fit with Pikes model as the longer the gap between menarche and FFTP the longer that breast tissue is at its maximal rate of tissue ageing. Li et al did however publish an earlier study in 2007 in which they found an increased risk associated with earlier age of menarche (≤11yrs compared to ≥14yrs RR = 1.3)(174).

A smaller meta-analysis, only including 9 studies, found that an older age at menarche was protective against both oestrogen and progesterone receptor positive (ER+/PR+) tumours and negative (ER-/PR-) tumours(175). These results will need to be replicated by further studies and some research will be require to identify biological significance of this is still finding. The majority of the evidence supports a relationship between age at menarche and breast cancer risk, as being a small but significant effect on risk.

Age at menopause is also an established breast cancer risk factor and some of the significant papers which established this are highlighted in a review by Parkin (2011)(111). One of these papers found that the risk of breast cancer was doubled in women who went through the menopause at age 55 years when compared women who went through it at 45 years old or younger(176). In 1996 the Collaborative Group on Hormonal Factors in Breast Cancer published a large re-analysis of 51 papers which found similar results to the above

study. They found that for every year older a woman was at natural menopause there was 2.9% (standard error (SE) 0.3) increase in breast cancer risk(118). In these large re-analyses it is important to remember they may not lack statistical power, but due to the large variety of papers used there is a substantial likelihood of heterogeneity, due to differing methodologies and study populations. A second paper by the same group published 2012 found the RR = 1.029 (95% CI: 1.025-1.032; p<0.0001) with every year older at menopause (figure 2.2)(173). This later paper suggested that the age at menarche seemed to have a larger effect on breast cancer risk than age at menopause. It is still not known why this was the case.

It is clear from the evidence above that the both the age at menarche and menopause can both contribute a small but significant increase in breast cancer risk. The reason for this is thought to relate to the resistance of the breast tissue itself, exposure to endogenous hormones and the rate of hypothetical breast tissue ageing.

2.2 Age at First Full Term Pregnancy and Parity

Evidence suggests a later FFTP can increase the risk of breast cancer developing in later life, which may relate to the developmental stage of the breast tissue; as prior to pregnancy breast tissue is in an undifferentiated state and after the FFTP the breast tissue is fully differentiated. When fully developed it is thought to be less sensitive to carcinogens and endogenous hormones(107). The relationship between breast cancer and age at FFTP has been confirmed in a number of both animal and human studies(108).

A prospective cohort study by Li et al (2007) discovered woman whose FFTP was at aged 35 or older had a significantly increased risk when compared to those aged ≤19yrs at FFTP; RR = 2.0 (95% CI: 1.1-3.7; p = 0.01)(174). This is in agreement with the results from the Californian Teachers Study, who found in their cohort with 10.5 years follow up women who were 35 years or older at FFTP had a 27% increased risk in comparison to those who

had their first child at 21 years or younger. They also identified a significant trend for increasing risk with later age at FFTP (p_{trend}= 0.002)(113).

Some research has shown that an earlier age at FFTP specifically protects against ER and PR positive cancers(175,177). This is biologically plausible as an earlier FFTP will decrease exposure to endogenous sex hormones, but once again the specifics of this association remain unclear.

Parity was first associated with breast cancer over 400 years ago by Ramizzini in a population of nuns, as mentioned above. In recent years there have been formal epidemiological studies which have confirmed this relationship between parity and breast cancer risk; these have shown that higher parity is associated with a lower cancer risk. It is important to note that the protective effects of parity and breastfeeding are very closely linked and they may have a confounding effect on one another, as it is hard to disentangle the two(115). The mechanism underlying this protective effect is not clearly understood but it has been suggested it may relate to decreased levels of circulating oestrogens and progesterone. Additionally there is thought to be increased amounts of sex hormone binding globulin (SHBG) and therefore less free oestrogens, as well as an increase in human chorionic gonadotropin (hCG)(113).

A collaborative analysis which studied women who never breastfed from 47 studies, found that with each birth there was a significant reduction in breast cancer risk by 7% (SE 1.0%; $p<0.0001$)(115). The reduced risk with increased parity was observed regardless of ethnicity, age or country of origin. This reanalysis has two major strengths firstly its large number of participants and secondly that it controlled for breast feeding.

In general it has been found that having children is protective in comparison to being nulliparous (RR = 0.91), irrespective of number of children (113). There has also been an a trend for decreasing risk with increasing numbers of children identified (P_{trend} = 0.003)(113).

There is some evidence that this protective effect is lost with age. For example in the Long Island Breast Cancer study cohort in women less than 65 years old parity was protective(OR = 0.7, 95% CI 0.48 -1.10). An effect that was once again found to be stronger in those with more children (≥ 4 children OR = 0.48; 95% CI: 0.25-0.9). However when women over the age of 65 were studied this protective effect was found to be statistically insignificant(106). When interpreting these results it is important to note that the effect of increasing age on risk may be confounding the protective effect of parity in these older women.

A recent meta-analysis found an association between ER/PR positive tumours and parity – which found an 11% decrease in the risk of an ER+/PR+ tumour with each birth(175). This relationship may relate to the relationship between breast cancer pathogenesis and endogenous hormone levels(177).

Both young age at FFTP and multiparity convey a small but important protective effect against the development of breast cancer, which has been established by numerous large studies and meta-analyses. These two factors may play a role in the increasing incidence of breast cancer in the developed world over the last 40 years. As there have been a great deal of societal changes which have led to women having children later in life and many women choosing not to have children at all(3). However nulliparity and late age at FFTP are not the sole contributors to this increased incidence and other factors are almost certainly involved.

2.3 Breastfeeding

Breastfeeding has various benefits for mother and child, one of these benefits is that it is a potentially modifiable breast cancer risk factor. However over the years there has been some inconsistency in the data on breastfeeding and breast cancer risk, with studies producing a wide range of results(115). The inconsistency may in part be due to the problems caused when trying to separate it from parity. There are several proposed mechanisms by which breastfeeding is thought to be protective including postponing ovulation, decreasing circulating oestrogens and progesterone, or increasing prolactin levels. It may also initiate the terminal differentiation of the breast epithelium, therefore allowing the breast tissue to be more resistant to potential carcinogens. Another proposed mechanism underlying this protective effect is the direct mechanical effect of the excretion of oestrogens and carcinogens during lactation(114).

Breastfeeding was investigated in a prospective cohort of 60,075 parous pre-menopausal women by the Nurses' Health Study (112). The relative risk of women who had ever breastfed was 0.75 when compared to those who had never breastfed; these results remained unchanged when adjusted for age at FFTP and multiparity. The length of time spent breastfeeding was not found to have any association with risk. In women with an affected first degree relative the protective effect was significantly increased– RR = 0.41 (95% CI: 0.22 – 0.75)(112). Suggesting breastfeeding may have added protective benefits in women with a family history of breast cancer.

However other studies have failed to reproduce these results, for example a study of breastfeeding in a Japanese population found that there was not enough evidence to support a protective effect(114); this was a systemic review which included 3 cohort studies and 5 case-control studies. Individually these studies found a range of results from null findings to an inverse relationship and only looked at small numbers within a small

population - for example one in particular only had 3 controls who had never breastfed(114). The above factors may have contributed to the inconclusive results of these individual studies and the systemic review.

Other larger studies have found results corresponding to those of the Nurses' Health Study, an example of one such study was a large re-analysis of 47 papers from 30 different countries(115). This large re-analysis however also found a relationship between duration and parity; every 12 months of breastfeeding conferred a 4.3% significant decrease in risk. There was also a significant decrease in risk (3.4%) for each child breastfed, all these results remained the same when adjusted for country, age, ethnicity and childbearing pattern (115).

A meta-analysis looking at the relationship between breastfeeding and tumour characteristics found that breastfeeding was associated with a decreased risk for both ER+/PR+ (RR = 0.95) and ER-/PR- cancers (RR = 0.91)(175). Therefore it is possible that the protective effect of breastfeeding differed from that of multiparity and age at FFTP. This is perhaps demonstrated by the number of different plausible mechanisms by which breastfeeding may exert its protective effect(177).

The literature on breastfeeding and breast cancer does suggest it exerts a small protective effect, but there are inconsistencies as to whether number of children and duration of breastfeeding alters this protective effect. There are many difficulties in getting a clear impression of the impact breastfeeding has on breast cancer due to the issues with data collection including recall bias and the difficulties disentangling breastfeeding from parity.

2.4 Hormone Replacement Therapy and Hormonal Birth Control

Prescribed hormonal preparations have been studied a great deal with respect to their effect on breast cancer risk. Hormone replacement therapy (HRT) is a well-established and

generally accepted breast cancer risk factor. The evidence as to whether hormonal birth control (HBC), including the oral contraceptive pill (OCP), contributes to breast cancer risk is less abundant, consistent and clear cut. These prescribed hormonal preparations introduce exogenous oestrogens and progestogens which alter the hormonal milieu of breast tissue. They are therefore thought to have the potential to increase breast cancer risk, as these hormones increase proliferative and developmental activity within the breast tissue.

Three large studies provided the basis for the association between breast cancer and HRT use - these were the Women's Health Initiative (WHI)(178–180), Collaborative re-analysis(110) and the Million Women Study(117). A double blinded oestrogen-progestin/placebo randomised trial was performed by the WHI study with 16,608 post-menopausal female participants, which aimed to assess the effect of HRT on cardiovascular and breast cancer risk over a period of 8.5 years follow up. However the trial was stopped early after only 5.6 years due to a recommendation by safety board, as the risk of developing breast cancer was deemed to outweighed the benefit of the trial continuing(179,180). This was because the safety limit of breast cancer occurrence among the group using HRT being reached. A preliminary analysis of the results demonstrated that women taking combined HRT had relative risk of 1.24 of developing invasive breast cancer (weighted $p = 0.003$). The publication of these results was accompanied by a global decrease in the use of HRT(181).

In the same time period the MWS was published and consisted of data from a large UK cohort(117). They found a 43% increase in breast cancer risk in women who had ever used HRT when compared to those who had never used HRT. Current HRT users had a 66% increased breast cancer risk; however this increased risk was attenuated within 5 years of stopping therapy. The MWS found the largest risk associated with the use of a combined preparation (RR = 2.00; 95% CI 1.88-2.12). However the follow up on this study was

extremely short at only approximately 2.6 years, this could have led to an inaccurate calculation of risk. This said the MWS did confirm the results of the WHI and the results of the MWS are summarised in *Figure 2.3*.

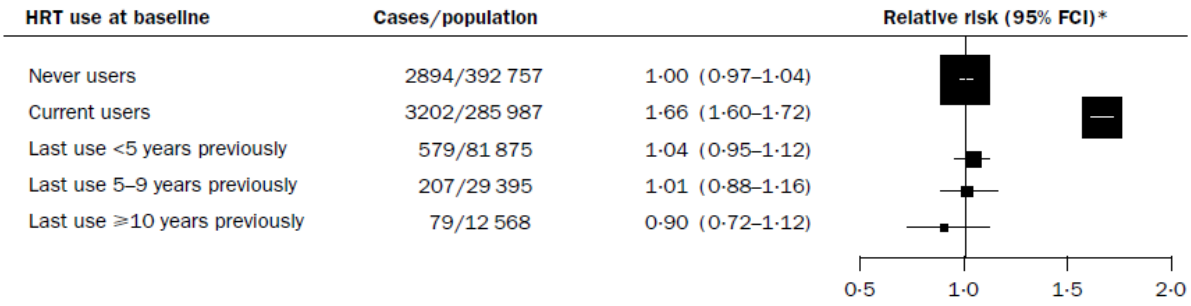


Figure 2.3 - Summary of the findings from the MWS (Adapted from the MWS -(116)). The relative risk of HRT use is highest in current users and decreases to that of the population/never users within 5-9 years of discontinuing therapy.

Prior to both of these studies the Collaborative Group on Hormonal Factors in Breast Cancer published evidence that HRT use was associated with an increase in breast cancer risk(110). They demonstrated that women who had ever used HRT had a 14% increase in breast cancer risk. This increased risk was further increased with longer duration of use. Once again current users were demonstrated to be at the highest risk with a 22% increase in risk. In agreement with the MWS, the Collaborative Group on hormonal factors demonstrated this excess risk was found be attenuated after 5 years of discontinuing HRT (5-9 years ago V.s. never RR = 1.01).

Shapiro et al (2011)(182) have published a series of articles criticising these studies, questioning their methodological validity and the biological plausibility of HRT increasing breast cancer risk. The authors claimed that the use of HRT by patients had declined unfairly due to the evidence published by the MWS, WHI and Collaborative reanalysis. It is important to note that several of the authors involved in the critical analysis of these studies had all at one point been employed by pharmaceutical companies responsible for the manufacture of HRT.

However contrary to the claims of Shapiro et al there is epidemiological data demonstrating a decline in worldwide breast cancer incidence which coincides with the decline in HRT use from 2002 (181). It could therefore be assumed that the decrease in HRT use may be one of the contributing factors to this decrease in incidence. For example in Germany the use of HRT declined by 50% between 2002-2005 and over the same period there was a 8.7% decrease in the incidence of breast cancer. Similar trends were noted in other developed countries including France, Belgium, Australia, New Zealand and Canada. There are most certainly other factors which will have contributed to the decline in incidence, however it is conceivable that a proportion of this decline is related to changes in HRT use(181).

Various studies have come to differing conclusions as to whether HBC increases the risk of breast cancer. In part this may be due to the various different hormonal preparations which are available and the changes in these preparations and their doses over time(120). Other factors could be also acting to confound the results as well including nulliparity, not breastfeeding, and age at FFTP – all of which are increased by the use of HBC.

A combined analysis of 54 epidemiological studies investigated the relationship between the OCP and breast cancer found that current users had a 24% increase in risk (95% CI: 1.15-1.33), which diminished to 7% (95% CI: 1.02 – 1.13) after 10 years of stopping use of the OCP. This analysis failed to find a relationship between risk and the preparation of OCP used or duration of use(118). These results have been replicated by a more recent study, showing current HBC use increases risk by 33% but they found no association between past HBC use and risk(121).

A meta-analysis of case-control studies investigating the use of OCP among pre-menopausal women concluded that ever using OCP caused a 19% increase in the risk of developing breast cancer(123). Stratifying women into groups dependent on parity or

whether HBC was used before or after FFTP did not significantly alter the results. It is important to note that the use of crude odds ratios due to the heterogeneity of the papers analysed may have led to an overestimation of risk by this analysis.

A systemic review analysed a number of recent papers and concluded OCP may increase the risk of developing breast cancer, despite a number of studies which failed to find any association between the OCP and breast cancer(120). There is some evidence that the use of the HBC could have a modest effect on breast cancer risk, however like HRT its effect is attenuated within 5-10 years of discontinuing treatment. It does however remain unclear if the duration of use, preparation and age at exposure to HBC has any influence on breast cancer risk. Trying to tease out this relationship may prove difficult due to the large number of preparations available, recall bias, and the issues disentangling it from the risk associated with parity and age at FFTP.

There is a considerable body of evidence including some large pooled analyses supporting increased breast cancer risk associated with a range of reproductive factors: including age at menarche, menopause and FFTP, parity, HRT and HBC use are generally accepted as contributing to breast cancer risk.

3. BMI and Dietary Influence on Risk

Despite the fact that BMI and diet are intrinsically linked, only BMI has been consistently associated with breast cancer risk. There is less consistent evidence regarding the influence of diet on breast cancer risk. This may in part be due to the issues caused by trying to quantify and record a person's dietary intake, as well as the variation in diet worldwide.

3.1 Effect of BMI on Post-Menopausal Women

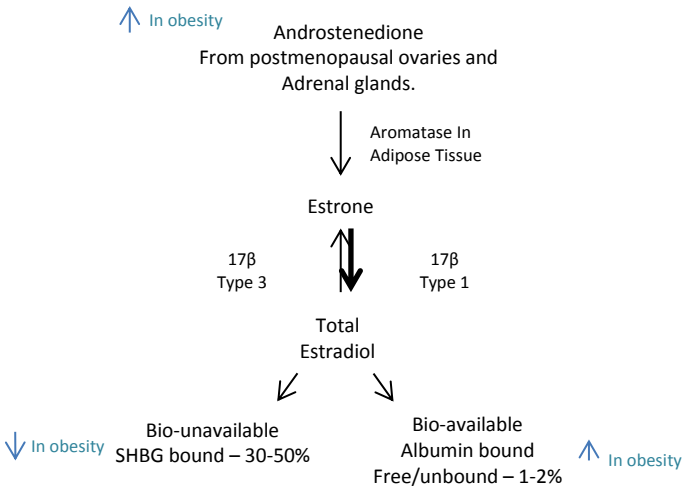


Figure 2.4 – This illustrates the oestrogen production pathway in postmenopausal women. Adipose tissue is vitally important in the conversion of Androstenedione to Estrone. The blue arrows denote any changes in homeostasis that are linked with obesity. This shows the potential for an increase in bio-available oestrogens in postmenopausal women with a high BMI. Adapted from Stephenson and Rose (2003) [66].

Body mass index (BMI) is a measure of obesity, which is calculated by dividing an individuals' weight in kilograms by their height in metres squared - ($BMI = \frac{Weight\ Kg}{Height\ m^2}$. The influence of BMI on breast cancer risk has been shown to differ dependent on menopausal status. Post-menopausally a high BMI is associated with an increased risk of developing breast cancer. The underlying biological mechanism relates to the conversion of androstenedione to estrone by adipose tissue in post-menopausal women to increase circulating levels of oestrogens. In women with a high BMI there is more adipose tissue so a greater capacity to increase circulating oestrogens. Obesity also decreases the levels of sex hormone binding globulin (SHBG). Together the increased peripheral conversion and decreased SHBG result in an increase in circulating bio-available oestrogens (*Figure 2.4*)(124,172,183). The use of HRT is thought to attenuate the effects of BMI on risk as it increases the circulating exogenous oestrogens far more than the level of endogenous oestrogen could be by the excess adipose tissue. So a high BMI is thought only to increase risk in post-menopausal women who do not use HRT(172).

There have been various different measures of obesity used across various different studies, and all have generally shown that obesity increases breast cancer risk in

postmenopausal women. BMI provides a more consistent measurement, when compared to waist circumference and hip to waist ratio as accuracy depends to a greater extend on the person taking the measurements. So for this reason BMI is often used as it seen as more reproducible despite having other problems(124,184).

Postmenopausal women who have a BMI in the highest quartile have a 57% increase in risk of developing breast cancer, compared to those in the lowest quartile, according to data from the WEB study(126). Lahmann et al replicated these results, finding women with BMI's in the highest quartile had a 54% increase in breast cancer risk (*Table 2.1*)(185). The use of HRT was found to attenuate this increase in risk; which is thought to be due to the endogenous hormone production being greater than the exogenous hormone in HRT, therefore the risk produced by HRT is no longer significant. A significantly increased breast cancer risk in postmenopausal women who had never used HRT was found by the MWS, which found women with a BMI >25kg/m^2 had a RR = 1.4(128). Another large study called the Pooling Project re-analysed data from a large number of published cohort studies(127) and found that postmenopausal women with a high weight that had not used HRT had a higher breast cancer risk.

There has also been speculation as to whether weight gain has any influence on risk, and there have been several papers which have found an association between increased risk and weight gain from the age of 20 (126,185,186). The conclusion of one such study was that for every 5kg of weight gained there is a 4% increase in breast cancer risk(126). These results must be interpreted carefully as this increase in risk may just reflect these women having a higher BMI, and therefore the risk could be associated with being overweight or obese and not the weight gain.

BMI (kg/m^2)[3]	*Multivariate-Adjusted RR (95% CI)*
<22	Reference
22.0-23.8	1.05 (0.67 – 1.62)
23.9 – 25.7	1.20 (0.78 – 1.85)
25.8 – 28.5	1.31 (0.86 – 2.01)
>28.5	1.54 (1.01 – 2.35)
P trend	0.023

Table 2.1 - Relative risk of breast cancer in postmenopausal women according to BMI. Adapted from the Malmo study, Lahmann et al (185).

The evidence above supports a relationship between a high BMI and the risk of developing breast cancer in postmenopausal women who have never used HRT. This is especially evident in women who fall into BMI categories which lie in the highest quartile (*Table 2.1*). However it seems this increased risk is completely diminished by the use of HRT, which is thought to relate to the levels of circulating exogenous and endogenous hormones.

3.2 Effect of BMI in Pre-Menopausal Women

However in premenopausal women the story is slightly more complicated with a number of studies reporting a decreased breast cancer risk in women with a high BMI. The biological reason underlying this observed relationship is poorly understood. It is thought that it perhaps relates to the irregular menstrual cycles associated with obesity; due to these irregular cycles these women will potentially have lower levels of circulating endogenous hormones(124), which are predominantly produced by the ovary during this stage of a woman's life(172,183). Lower levels of circulating hormones will in turn have an effect on Pikes hypothetical tissue ageing process. So the lower levels of hormones may protect the breast tissue by slowing the accumulation of cellular changes associated with Pikes ageing process.

Both the Pooling project(127) and the MWS(128) found a protective relationship between premenopausal obesity and breast cancer. Research by the pooling project found the relative risk was 0.58 when pre-menopausal women >80kg were compared with those <60kg(127). A borderline significant trend ($p = 0.05$) for decreasing risk with increasing

BMI was uncovered in premenopausal women by the MWS(128). Current evidence indicates that a high BMI may be protective against breast cancer in premenopausal women, which is the opposite of what has been found in postmenopausal women. However further work is required to fully establish this relationship and to uncover the biological mechanism underlying it.

3.3 Diabetes Mellitus

A high BMI is closely associated with type two diabetes mellitus (DM) and recently evidence has emerged which suggests that type two DM is an independent risk factor for breast cancer in postmenopausal women(187,188). A proposed mechanism responsible for this increased risk involves both a direct mitogenetic effect in the breast epithelium by insulin; and an indirect effect via the increasing the levels of bio-available oestrogens by lowering the levels of SHBG (see *Figure 2.4)*(172).

A meta-analysis by Larsson et al (2007) which looked at 20 published papers supported the hypothesis that DM contributes to breast cancer risk; this study concluded that there was a 20% increase in risk in women who had type 2 DM when compared to those who did not(188). However some of the studies included did not distinguish between the two types of DM, which have very different pathological processes. The fact that the majority of diabetes is type two may have meant that including both types lead to an underestimation of risk. This is a relatively new area of breast cancer epidemiological research and in the coming years it will be interesting to see the results of ongoing research.

3.4 Diet

A large amount of research has been carried out to try and associate dietary factors with breast cancer risk, as this is a potentially modifiable risk factor which could be used preventatively and may provide the key to explaining observations made by migration

studies. Various foodstuffs have been investigated including: soy intake, red meat intake, cooking methods, fat intake, fruit and vegetable intake, dairy and egg intake. Diet may modify risk by various mechanisms including DNA repair, DNA adducts, detoxification, carcinogens and have effects on oestrogens(129).

However there are major methodological issues associated with these studies as quantifying diet over a lifetime is an extremely difficult task to undertake. Issues such as recall bias are highly problematic, as the majority of people are unlikely to remember their dietary intake over a long period of time. Recall bias could be present in other studies but, events relating to reproductive history are major events in a woman's life which are far more likely to be recalled and with greater accuracy. In an attempt to overcome the issues of recall bias the Food Frequency Questionnaire (FFQ) and the 7 day diet diary (7DDD) are often used in this type of study. The FFQ asks participants to give information on how often they consume particular foods with simple measures of portions; whereas the 7DDD requires participants to list everything they have ate and drank over a 7 day period(129). There are advantages and disadvantages to the use of both methods of quantification; however the FFQ is more commonly utilised as it is slightly simpler.

Due to the lower incidence of breast cancer in Asia it has been suggested that the high soy content in the Asian diet may have a protective effect(138). One meta-analysis of 18 studies found some evidence in support of a protective role of high soy consumption against breast cancer in both pre- and post-menopausal women. Both authors did however conceded that these results should be interpreted cautiously(138) because of the availability, quantity and heterogeneity ($\chi^2 = 41.06$; $p = 0.002$) of the available data. There is currently not enough evidence to fully support a protective role for soy and soy products against breast cancer. This may partly be due to difficulties in assessing soy intake, and the lack of large scale studies.

High intake of red meat is thought to increase breast cancer risk. This is thought to be especially true if the red meat is cooked at a high heat as this may increase the number of polyaromatic hydrocarbons (PAH) and volatile chemicals which can be produced during cooking(130,134). There has not been a consistent link established between risk and red meat; with some papers finding it increased risk (130,134) and others failing to find an association(131,132). There is a lack of consistent evidence which associates any modest increase in breast cancer risk with a high red meat dietary content or how it is cooked.

The consumption of a diet high in fruit and vegetables has been associated by some with a protective effect, which is thought to be due to their antioxidant properties. Once again the evidence remains inconsistent and the relative risks vary widely between different studies. In some studies only high fruit consumption has been associated with a decreased risk(136). Others have found small significant decreases in risk with high fruit and vegetable intake(135), but others failed to find a relationship(136,137). There have been vast amounts of work done looking at the relationship between risk and red meat, fruit and vegetable intake; however this has failed to produce conclusive evidence that there is a relationship between these foodstuffs and breast cancer. This is may be in part due to difficulties with quantifying dietary intake, data collection and defining the best time frame to collect data in. It is currently still unknown if a woman's diet is more important during puberty, at menopause, or prior to FFTP in terms of breast cancer risk.

It has also been suggested that dietary fat intake may elevate the endogenous oestrogen levels and therefore predispose to breast cancer(129). Holmes et al (1999) failed to find a significant relationship between high fat diet and risk, but they identified that a low fat diet (<20% energy from fat) may be protective. However finding women who consume a diet that low in fat is extremely rare, therefore finding enough participants to carry out a study

on this would be extremely challenging(133). There have been multiple studies to date which have failed to find a relationship between the two(189,190).

Evaluating all the information above there is not enough consistent evidence to suggest a link between any dietary factors and breast cancer risk. This does not mean there is not an association between breast cancer and diet; the lack of relationship to date may relate to the methodological issues faced when carrying out this type of study. Additionally the majority of studies fail to take into account BMI, which is intrinsically linked to diet and may be influencing the results of the above studies.

4. Alcohol Consumption

There is now a body of evidence which supports a moderate increase in breast cancer risk with alcohol intake. The biological mechanism which is thought to underlie this is that a moderate alcohol intake can increase the levels of circulating oestrogens, due to either an increased secretion or decreased clearance(144). This is supported by the results of Dorgan et al who carried out a blinded controlled feeding study. Women who consumed 15g of alcohol (approximately 2 UK units or 250ml glass of wine) or 30g (~3.5 units) of alcohol daily for 8 weeks - Dorgan et al found that this increased their serum estrone sulphate levels by 7.5% and 10.7% respectively(191). A second proposed mechanism is that acetaldehyde, a product of alcohol metabolism, may induce the production of reactive oxygen species which can cause cellular damage (192).

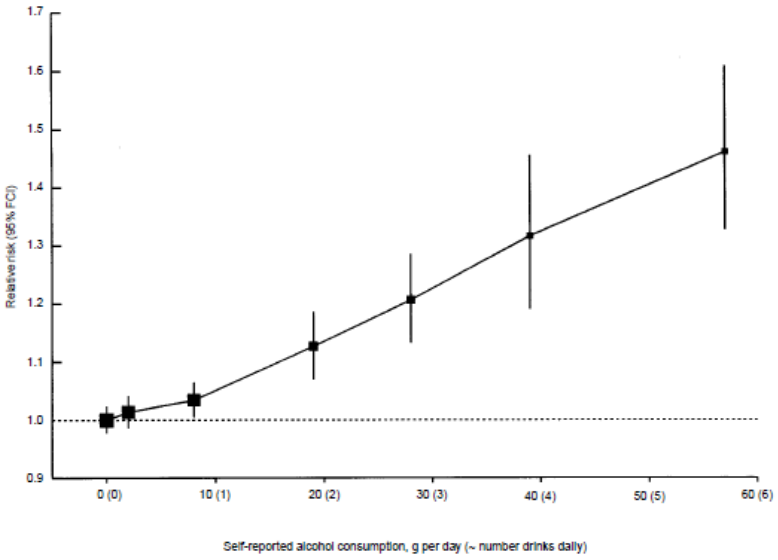


Figure 2.5 - Relative risk of breast cancer increases with increasing alcohol consumption. Adapted from Hamajima et al (139).

A positive relationship between alcohol intake and breast cancer risk has been confirmed by a number of studies(139,144,192–194). A large re-analysis of 53 papers concluded drinking 4-5.5 units (35-44g or half a bottle of wine) of alcohol daily causes a 32% increase in breast cancer risk. Drinking over 45g daily further increased this risk by a further 14% to 46% (*Figure 2.5*)(139). A second smaller study found for an increase of 10g of alcohol a day there was an associated 9% increase in risk (95% CI: 1.04-1.13)(144). However there is currently still no evidence to support that the type of alcoholic beverage influences risk(139,144,192).

Even a moderate increase in alcohol intake (10g/d which is ~1 drink a day) is thought to increase breast cancer risk(194). The mechanism which underlies this could relate to an increase in endogenous oestrogen, or an increase in reactive oxygen species. There are issues with studying alcohol intake as participants tend to under report consumption and there is a poor public understanding of the unit. These factors may mean that the risk is being under-estimated by current studies, and that the risk conferred by alcohol consumption could be greater than currently estimated.

5. Smoking and Environmental Pollutants

5.1 Smoking

A panel of experts assessed all the available evidence with regards to breast cancer and smoking concluded that there was sufficient evidence to link breast cancer and active smoking, but not passive exposure(143). It is thought that the carcinogens in tobacco smoke may be transported by lipoproteins and stored in breast adipose tissue. These compounds stored in this adipose tissue are later metabolised and activated(195). Metabolites of tobacco smoke have been found in breast fluid of smokers, which supports the above mechanism(196). These active metabolites are thought to cause damage to the breast tissue and have carcinogenic effects.

There have been a number of positive associations found(142,195–201), some null results(139,169,202,203) and some studies have shown an inverse relationship between breast cancer and smoking. One of these papers found a 32% (95% CI: 1.10 – 1.57) increase in breast cancer risk in current smokers; and greater exposures to tobacco are associated with a higher breast cancer risk(196). Many papers have suggested that this risk was also greater the younger the woman started smoking or if they began smoking before FFTP(142,169,195,196,199,201). This may be because the breast tissue is undifferentiated prior to the FFTP, which means it is more sensitive to the effects of the chemicals release by cigarette smoke; this phenomenon has been confirmed in animal studies(169). Trying to separate smoking at a young age and smoking for a long time is very difficult, but currently both are thought to be associated with risk(141,169,196–198).

Genetics may modify the level of breast cancer risk associated with smoking, as N-acetyl transferase 2 and other proteins play a critical role in detoxifying the carcinogenic compounds released from tobacco smoke (140). Individuals homozygous for a variant of the N-acetyl transferase (NAT) 2 gene are known as slow acetylators, and there is evidence

that these women are at a greater breast cancer risk if they smoke(140). This is an on-going area of research but it does highlight that the interaction between genes and the environment can alter risk.

There is however less consistent evidence as to whether passive smoking has any relationship with breast cancer risk; however the reason for the lack of consistent evidence may relate to the difficulties in assessing passive smoke exposure(204). Many people have chosen to assess passive smoking by assessing whether women have ever lived with a smoker – however at best this is a crude measure of cigarette smoke exposure as it does not take into account smoke exposure outside the home. A Long Island study showed women who lived with a smoking partner for >326 months had an increased relative risk of 2.10 (95% CI: 1.47 – 3.02)(205). The authors of this paper conceded that despite this evidence there was still no strong evidence associating breast cancer and passive smoke exposure. Other studies have failed to find a relationship between the two (RR = 0.9; 95% CI: 0.83 – 1.2)(201).

As the evidence currently stands active smoking has been accepted as causing an increased risk of breast cancer. However there is still some uncertainty as to whether a high BMI alters this association between active smoking and risk(206). Additionally the strength of the association between the timing of active smoking and duration of active smoking have on breast cancer risk remains unknown, but separating the two for future studies will be difficult.

There is not enough evidence to support a relationship between passive smoke exposure and breast cancer risk. Some studies have found that passive smoking may influence breast cancer risk, but the majority of studies have found little to no association between breast cancer and passive smoking. The reason for this lack of evidence may be due to the difficulties in quantifying passive smoke exposure.

5.2 Environmental Pollutants

Exposure to various environmental pollutants has been widely investigated to determine if they are associated with breast cancer risk. However, despite the vast amount of research in this field there has been no conclusive evidence linking the two. Once again this lack of consistent evidence may relate to the difficulties of measure a persons' lifetime exposure to an environmental pollutant.

Breast cancer incidence has been demonstrated to be higher in industrialised and urban areas, which has led to a variety of studies looking at the contribution of air pollutants to breast cancer risk(167). One such study was the WEB study (Western New York exposures and breast cancer), which used a surrogate marker of harmful atmospheric chemicals like PAH and benzene called total suspended particles (TSP)(167). There was a non-significant trend towards increased risk with increased TSP exposure in pre-menopausal women who were exposed at menarche and post-menopausal women exposed at time of FFTP. This study failed to explore the contribution of cumulative exposure to TSP to breast cancer risk. Other studies which used TSP or PAH exposure have also failed to find an association with breast cancer(207,208).

Studies have investigated other exposures including one US study, which looked at the correlation between atmospheric nitric oxide and breast cancer incidence(165). A correlation between the two was identified – and the states with the highest emissions had the highest breast cancer incidence(165). However this did not take into account any other factors which contribute to breast cancer risk such as socioeconomic status, reproductive factors and ethnicity. A second study replicated these results in a Canadian population finding that higher NO_2 exposure correlated with increased breast cancer risk(166). Unfortunately this study used crude predictive measurements of exposure and some controls had other cancers, therefore these results need to be interpreted with caution.

It is thought these environmental pollutants may promote DNA damage, tumour growth and increase the susceptibility of tissues to undergo malignant transformation(209). Unfortunately there is still a lack of evidence to conclude that certain environmental exposures contribute to breast cancer risk; however there are indications that this may be the case and research is ongoing.

6. Ionising Radiation

Evidence from women exposed to therapeutic radiation (for example Hodgkin Lymphoma treatment) and Japanese atomic bomb survivors have established ionising radiation as a breast cancer risk factor(148). Exposure to ionising radiation prior to the age of 20 is associated with the highest risk of developing breast cancer and displays dose dependent relationship(149).

Zheng et al (2002) demonstrated that postmenopausal women exposed to therapeutic doses of radiation on 6 or more occasions had an increased breast cancer risk – odds ratio (OR) = 2.5. If these women were exposed before their 20th birthday then they had a higher risk than those exposed aged 20 or older - < 20 yrs OR = 2.1; >20 years OR = 1.4(150). This case control study failed to show any associations between diagnostic doses of radiation and breast cancer risk. For example the risk of breast cancer in women who had had 12 or more mammograms compared to those who had less than 6 was not significantly different - OR = 0.9 (95% CI; 0.6 – 1.3)(150).

The case control study by John et al is in agreement with this finding that women who had ever versus never experienced diagnostic radiation exposure had no increase in risk; OR = 0.99 (95% CI; 0.88 – 1.11) - this result was consistent regardless of whether these women where high or population risk of developing breast cancer(210). On the other hand any exposure to therapeutic doses of radiation has been associated with an increased risk in

comparison to women who have never had this type of medical radiation exposure; OR = 1.72 (95% CI; 1.70 – 2.55)(210).

The relationship between radiation exposure and breast cancer risk in BRCA 1/2 and p53 mutation carriers is thought to differ as they do not have the ability to repair certain types of DNA damage, as discussed above, which may be caused by even small diagnostic doses of ionising radiation(211). It is therefore believed that even diagnostic doses of ionising radiation (e.g. mammogram dose = approximately 0.4mSv) may increase breast cancer risk in these women; however there is little evidence currently to support this theory. One paper examining this phenomenon found that there was an increased risk in these women when exposed to diagnostic radiation, especially at a young age. When a comparison was made between no ionising radiation exposure and diagnostic radiation exposure in women under 30 years old the HR was 1.90 (95% CI; 1.20 – 3.00). Again there was a correlation between higher doses of radiation and increased breast cancer risk, as previously established in non-BRCA mutation carriers(211): estimated dose <0.0020 Gy HR= 1.63, ≥0.0020-0.0065 Gy HR = 1.78, ≥0.0066-0.0173 HR = 1.75, and ≥0.0174 HR = 3.84.

Mantle field radiation is used in the treatment of Hodgkin Lymphoma; the breast cancer risk in women who have undergone this treatment is 75 times that of the general population(150). There are several factors which modify this breast cancer risk such as time since treatment (latency approx. 15 years), age when treated, and dose(151). Exposure to this high dose radiation is proposed to cause loss or gain of function mutations and chromosomal rearrangements such as deletions and translocations, which act as an initiating step in the process of malignant transformation(151). Another study carried out by Basu et al (2008) found in their cohort of 398 women diagnosed with paediatric Hodgkin's Lymphoma that 25 women developed invasive breast carcinoma; and found that in their patient population there was a 37 fold increase in breast cancer risk(212).

Additionally they found that those women diagnosed with Hodgkin's Lymphoma before the age of 12 had the highest risk of developing breast cancer, this was thought to relate to the developmental stage of the breast at this age(212).

Exposure to high dose ionising radiation is a well-established breast cancer risk factor, which is an important consideration when assessing individual risk as recognised by both the UK NICE guidelines and the European EUSOMA guidance. The exposure to low dose radiation, such as a mammogram, is not thought to increase the risk of breast cancer developing in later life. However this is not thought to be case in women with familial breast cancer carrying BRCA1/2 mutations as they are less well equipped to cope with DNA damage. However these investigations are ongoing and it is still not clear if BRCA1/2 mutation carriers have an increased risk at lower doses of radiation.

7. Ethnicity and Socioeconomic Status

The highest breast cancer rates are associated with Caucasian women and women with a higher socioeconomic status (SES). Why this is the case is still unknown, but it is thought to be a complex combination of social, cultural and genetic factors.

Studies from the USA show the highest breast cancer incidence is in white women and the lowest in American Indians/Alaskan Natives (see *Table 2.2*). When compared to white women all other ethnic groups have a lower breast cancer risk, but only women of African-American origin have a significant reduction in risk (HR = 0.85; 95% CI: 0.73-1.0)(119). A study which was conducted on the UK population found that in comparison to the Caucasian population the South Asian population had a lower risk of developing breast cancer (HR = 0.81; 95% CI: 0.68-0.95)(152).

Ethnicity	*Incidence of breast cancer per 100 000*
White	141
African American	122
Asian/Pacific Islanders	97
Hispanic	90
American Indians/Alaskan Natives	58

Table 2.2: Breast cancer incidence rates associated with different ethnic groups in the USA. Results adapted from Chlebowski et al (2005)(119).

SES and ethnicity can be linked; and one paper demonstrated that white women from the highest SES group had the highest breast cancer incidence, whereas African-American women from the lowest SES group had the lowest incidence of breast cancer(154). Why this is the case is not well understood, however it may relate to social factors such as HRT use, age at FFTP or to the utilization of screening(154,213).

Irrespective of how SES was calculated (educational status, income, community or individual area) women from a higher SES group always have the highest breast cancer incidence, and also the best survival rates(153,155–157). A 20% increase in breast cancer risk was demonstrated by Robert et al when they compared women living in areas with the highest SES with the lowest(157). A different study found similar results (15% increase in risk) despite estimating SES using educational status(156). The reasons for the difference in incidence between different SES categories may be explained by behavioural differences such as higher alcohol consumption, later age at FFTP, or lower parity which are all established risk factors in their own right - alternatively the disparity may be due to other, perhaps unknown, environmental and genetic factors.

8. Mammographic Density

Mammographic density is an established breast cancer risk factor which may have an association with the low penetrance polymorphisms discussed above, but it is not strictly an environmental factor. However as it is an important contributor to risk it shall be discussed here. Mammographic density describes the appearance of the breast on a

mammogram and refers to the composition of the breast tissue. It describes the proportions of stromal and epithelial tissues which appear white on the mammogram; and fatty tissue which has a dark appearance(158,214). Therefore a dense breast has a greater the proportion of epithelial and stromal tissue within it.

High mammographic density was first hypothesised to be a risk factor in 1976 by Dr J Wolfe; who devised a qualitative assessment method known as Wolfe grades (N, P1, P1 and DY) for the assessment of breast density(161). The issue with this grading system is its dependence on the reader and a great variability between readers has been noted. Initially the increased risk associated with increased breast density was assumed to be due to masking bias, as dense tissue makes it more difficult to identify tumour. However more recent work has reassessed the relationship between risk and density, and has found a strong association between increased density and higher breast cancer risk (215). It is now generally accepted that a high mammographic density (>75%) imparts a 4 to 6 fold increase in breast cancer risk, when compared to women with a lower mammographic density(158,159,162,214).

A meta-analysis which looked at 42 studies found that regardless of the method used to measure density (e.g. Wolfe grade or percentage density) there was an increased risk associated to higher breast densities (summarised in *Table 2.3*)(162). It found that there was a relative risk of 4.64 (95% CI; 3.64 – 5.91) when women with >75% density were compared to those with <5% density(162).

Density Vs <5%	*RR (95% CI)*
5-24%	1.79 (1.48-2.16)
25-49%	2.11 (1.70 – 2.63)
50-74%	2.92 (2.49 – 3.42)
>75%	4.64 (3.64 – 5.91)

Table 2.3 - Summary of the results published by McCormack and Silva, showing that with increasing mammographic density there is an increase in breast cancer risk. Adapted from McCormack and Silva (162).

Other smaller studies are in agreement with the large meta-analysis. For example Boyd et al found a RR of 5.86 (95% CI 2.2 – 15.6) when comparing >75% density to 0%. This relative risk was adjusted for BMI, which allowed the authors to conclude that breast density and BMI are independent risk factors(159). A study conducted within a Spanish population concluded that mammographic density was a risk factor for breast cancer in general, as the odds ratios were found to be similar for both DCIS (OR = 3.47; 95% CI 1.46 – 8.27) and invasive cancers (OR = 2.95; 95% CI 2.01 – 4.35)(216). When looking specifically at receptor status Tamimi et al concluded that mammographic density was a risk factor for both ER and PR positive and negative tumours(163). These results confirm that high mammographic density is a risk factor for breast cancers; however it does not predispose to any particular type or tumour characteristic.

There are a lot of different hypotheses as to why breast density has an impact on breast cancer risk; these include higher circulating hormone levels, elevated growth factors, simply increased amounts of glandular parenchyma (therefore increasing the probability of developing a breast cancer) or unknown tumorigenic stromal factors (214). Discovering which, if any, of these mechanisms are responsible for the relationship between risk and density is an active field of research. Results emerging from this field include the nested case control study by Tamimi et al. This study is part of the Nurses' Health Study and explored the relationship between circulating hormone levels and density. Both were found to be independent risk factors, as relative risk between the highest and lowest density quartiles did not significantly change when adjusted for hormone levels; RR = 3.8 (95% CI: 2.2 – 6.6; p trend <0.001)(163). These results are not suggestive that the increased risk associated with density is related to hormone levels, but it is too early a stage to rule out this mechanism. Pinto-Pereira et al (2011) demonstrated that cancers tend to develop in areas of focal and persistent mammographic density suggesting that the high epithelial

and stromal content of a dense breast is the underlying reason for increased breast cancer risk(217).

One potential mechanism which may underlie the increased risk associated with high breast density is that there are unknown stromal factors acting in such a way that can lead to the development of breast cancer(214). Therefore in an attempt to look at the stromal factors present in an established breast cancer the experiment in Appendix 5 was carried out. The aim of this initial experiment was to extract RNA from formaldehyde fixed paraffin embedded tissue, which would allow analysis of gene expression and comparison between normal, reactive and tumour stromal tissue. However due to the fixing process the quality of the RNA was too low to allow any further analysis as certain quality control standards must be met to allow real-time polymerase chain reaction (RT-PCR) or microarray analysis. Nonetheless the concept of further investigating the gene expression within the stromal tissues surrounding cancers would be of interest to pursue further in the future; as this has the potential to link into both the genetic risk factors, which may have altered expression, and may also differ depending on distance from the tumour. To do this in the future would likely require RNA extraction from fresh frozen tissue, which brings with it a number of logistical issues; however a protocol for collecting tissue in this way was also written and can be found in Appendix 5. Unfortunately due to time constraints and the problems with identifying suitable candidates further molecular genetic analysis was not possible at this time. In the future analysis of the gene expression, particularly the low penetrance genes identified to effect breast cancer risk, would provide an interesting focus of research and perhaps allow the examination of the mechanisms by which breast cancer develops in further depth.

Irrespective of the underlying biological mechanism the evidence clearly points to mammographic density having a strong association with breast cancer risk. It is now widely

accepted that having high density breasts causes a 4 to 6 fold increase in risk, which is an effect of the high density itself and not due to masking bias. Some estimates predict that high breast density could account for up to 30% of all breast cancer cases(161).

9. Gene-Environment Interactions

During the previous chapter the effect of gene-environment interactions on risk were touched upon; for example *ZNF365, LSP1* and *RAD51L1* have all been associated with mammographic density(74,81). This demonstrates a relationship between genetic and environmental risk factors which act together to alter risk. It is also becoming clear that common processes or pathways are involved as the proposed mechanisms by which multiple genetic and environmental risk factors confer risk. For example oestrogen signalling and DNA repair pathways have been implicated in the plausible mechanisms for various factors discussed in this literature review. Therefore breast cancer research has begun to investigate interactions between genes and environment, in the hope that the poorly understood underlying biological mechanisms which are involved can be elucidated. This is in turn is hoped to have preventative and risk prediction benefits(218).

Far fewer studies have been published on the subject of gene-environment interactions as this is a newly developing field in breast cancer research. However the focus of the published articles on this subject have been on reproductive risk factors; particularly age at menarche, age at natural menopause, parity and HRT use(8,219–222). As of yet there is not a conclusive body of evidence demonstrating a positive correlation between any of the genetic and environmental risk factors, with many authors producing null results(8,220,221,223).

There have however been some positive findings, but in many cases these results have not yet been replicated. One such study by Nickels et al(219) found associations between several SNPs and environmental factors in their large collaborative study with 34,793 cases

and 41,099 controls. In this study they found that there were associations between *LSP1* and parity, *NOTCH2* and parity, and *CASP8* and alcohol. There was also weak evidence that the risk conferred by *RAD51L1* was modified by HRT use(219). On the other hand a previous paper published as part of the MWS failed to find any significant associations between *FGFR2, TNRC9, MAP3K1, CASP8, LSP1* and *ATM* and a range of environmental risk factors(220). However, these authors did find a relationship between higher alcohol consumption and *CASP8*; finding that there was an increase in breast cancer risk per allele in women who had one or more drinks a day in when compared to women who had less than one alcoholic drink a day. In addition to relationships between *TNRC9* and age at menarche, age at menopause and HRT which were all of borderline significance(220).

Other authors have found differing associations, for example *CASP8* has also been associated with a decrease in menopausal age by 1.12 years for each A allele a participant had in a small cohort(222). However in a second verification cohort these results could not be replicated, and there was no information provided on the effect on breast cancer risk. Nonetheless it could be inferred that this would increase risk, as an older age at menopause has been found to increase an individual's relative risk of breast cancer(111). An earlier paper by the same group did however look at how these interactions could modify breast cancer risk, and found that women with a larger gap between menarche and FFTP had an increased risk of breast cancer if they were heterozygous or homozygous for the G allele of 5p12 (*PDCD9*)(224).

There seems to have been a particular interest in the study of *FGFR2* and its interactions with environmental risk factors; this may be because it has been consistently replicated and seems to confer the largest relative risk of all the low penetrance polymorphisms identified. There is some evidence to support that *FGFR2* may increase risk of HRT therapy, and in particular oestrogen only therapy(225). However other studies have been unable to

replicated this finding(220,226). The risk associated with *FGFR2* has also been found to be modified by age at menarche, parity and a BMI>25 in post-menopausal women(226). The interactions identified between *FGFR2* polymorphisms, reproductive factors and ER positive tumours has led to hypotheses that these polymorphisms may influence breast cancer risk via hormonal pathways(225).

It has been proposed that some genes act to modify breast cancer risk only in combination with environmental factors; these genes will therefore be missed by GWAS and other such genetic studies. One group is therefore utilising gene-environment interactions to identify novel genetic risk factors(227). In this way two SNPs on Runt related transcription factor 1 (*RUNX1*) were identified in association with BMI to decrease risk with increasing BMI. However for these results to be replicated it would require huge numbers and a great deal of epidemiological and genetic information.

Finally, very little attention has been paid to the higher penetrance risk genetic mutations associated with breast cancer as these may also interact with their environment. Dennis et al looked at alcohol consumption and *BRCA* mutations to see if high alcohol consumption influenced breast cancer risk. Unfortunately this paper had very few *BRCA1* (n = 10) and *BRCA2* (n = 33) mutation carriers, but their findings did suggest an increase in breast cancer risk in *BRCA2* carriers who consumed more alcohol(228). However further studies with larger numbers of BRCA mutation carriers would prove interesting, and help to identify if environmental factors modify the risk associated with these high penetrance mutations.

By looking at gene-environment interactions a great deal of information on biological mechanisms may be gained and there is also the opportunity to identify novel genetic risk factors. However as this is a developing field there is still limited evidence available and apparent issues with reproducibility(229). In addition the current evidence concentrates heavily on reproductive factors with fewer studies investigating other risk factors such as

BMI, alcohol, smoking and radiation exposure. The patient populations which have been studied to date also have a heavy emphasis on Caucasian populations, but there is already evidence that ethnicity influences breast cancer risk(119) therefore it is feasible the gene-environment interactions would differ in these populations. Finally the majority of the research is looking for interactions between any gene and any environmental factor, without taking into account what is already known about the proposed biological mechanisms thought to underlie risk in each case. For example by hypothesising that the *ESR1* gene may interact with the reproductive risk factors, alcohol and BMI to alter risk - as all are thought contribute to risk via hormonal mechanisms - would perhaps be more likely to produce reproducible associations between genes and environment. It is clear that by using the current knowledge on individual breast cancer risk and prognostic factors, and applying it in a more holistic way to look at the patient as combination of their genes and environment, there is an opportunity to look at breast cancer risk in a new way. This different perspective on breast cancer risk and prognosis may provide insight into underlying biological mechanisms and there contribution to risk, as well as identifying new genetic risk factors.

10. Conclusion

There are a huge number of articles which have been published that investigate the various different environmental factors that have been proposed to contribute to breast cancer risk. Evidence for some risk factors, such as the use of hormonal preparations, is extremely consistent and has led to them becoming generally accepted as contributing to breast cancer risk. On the other hand evidence for other risk factors is far from consistent and no consensus can be reached as to whether these things influence risk at all, an example of such a factor would be diet. However, the reason for this lack of evidence may be due to the difficulties faced by researchers when trying to design studies to evaluate these

Risk Factor	RR	Reference
Age at Menarche**:** Every year <13 years at menarche	1.05	Collaborative Group on Hormonal Factors (2012)(173)
Age at Menopause**:** every year older at menopause	1.029	Collaborative Group on Hormonal Factors (2012)(173)
Age at FFTP**:** ≤19years Vs ≥35years at FFTP	2.0	Li et al (2007)(174)
Parity**:** Parous Vs Nulliparous	0.91	Ma et al (2010)(113)
Breastfeeding: Ever Vs Never	0.75	Stuebe et al (2009)(112)
HRT: Current Vs Non-user	1.22-1.43	Collaborative Group on Hormonal Factors (1997)(110); Millions Women Study Collaborators (2003)(117)
HBC**:** OCP Current Vs Non-users	1.24	Collaborative Group on Hormonal Factors (1996)(118)
BMI: Postmenopausal women highest Vs lowest quartile BMI	1.54-1.57	Lahmann et al (2003)(185); Han et al (2006)(126)
BMI: Premenopausal women highest Vs lowest group	0.58	Brandt et al (2000)(127)
Diabetes Mellitus: Type 2 DM Vs No diabetes	1.20	Larsson et al (2007)(188)
Alcohol Consumption: >4-5.5 units of alcohol daily Vs no alcohol	1.32	Hamajima et al (2002)(139)
Active Smoking: Active smoking Vs non-smoker	1.32	Reynolds et al (2004)(196)
Ionising Radiation: Therapeutic radiation Vs no medical radiation exposure	1.72	John et al (2007)(210)
SES: Highest Vs Lowest SES groups	1.15-1.20	Webster et al (2008)(156); Robert et al (2004)(157)
Mammographic Density: >75% density Vs <5% density	4.64	McCormack and Silva (2006)(162)

Table 2.4 – Summary of the relative risks attributable to each of the environmental factors which this thesis has found to have good evidence of association with breast cancer.

environmental factors. In addition to the effort involved in running these studies and numbers of patients required to be recruited for this type of study to reach significance.

This brief literature review demonstrates that there are a number of environmental factors which contribute to risk some of which are potentially modifiable and could be used preventatively: for example obesity, smoking, alcohol intake and breast feeding. The best established risk factors include breast density, age at menarche, menopause and FFTP;

parity; obesity (BMI); use of hormonal preparations; history of active smoking; ionising radiation; alcohol consumption and SES (Summarised *Table 2.4*).

There is less evidence which support a role for diet, environmental exposures and passive smoke exposure in breast cancer. However none of these factors have been ruled out as playing a potential role in risk and research is ongoing.

The factors which have been discussed in this chapter are all potential risk factors, however alone none of these risk factors are solely causative – perhaps with the exception of ionising radiation exposure. These risk factors contribute a small to moderate relative risk, which when combined with other environmental and genetic risk factors cause a woman to reach a threshold of risk and develop breast cancer. Additionally it is believed that there are also interactions between the genetic factors outlined in the previous chapter and the epidemiological risk factors reviewed above. However this is a relatively new area of research in the breast cancer field so there is a limited number of high quality research papers published on this subject. It is clear that there is now evidence emerging that these gene-environment interactions have the potential to modify breast cancer risk. One reason for this may be that both the genetic loci and epidemiological risk factors are converging in similar pathways to cause this increase in risk. Therefore by improving our understanding of these interactions we can uncover the biological mechanisms involved in breast cancer which are currently fairly poorly understood.

It is therefore clear that for the majority of breast cancers, with the exception of some familial cancers, there is a complex interplay between genetics and environment that result in its development. So by improving our understanding of not only these risk factors as single entities but as a whole and as part of complex biological machinery then we can understand an extremely common yet complex and heterogeneous disease better.

Environmental Factors which Alter Risk of Recurrence and Prognosis

1. Introduction

Breast cancer is a highly prevalent disease with over a million new cases reported worldwide each year(3). However the screening and treatment of breast cancer is improving and in the US alone there are 2.3 million breast cancer survivors(230). Patterson et al estimates that women diagnosed with breast cancer in 2009/10 will have a 5 year survival of 89% and 10 year survival of 82%(231). These predictions by Patterson et al are also in line with the findings of the survival analysis, which is outlined in the next chapter. This has led to an increase in the quantity of research into factors which influence disease prognosis. There is a special interest in modifiable environmental prognostic factors, which can be used to reduce the rates of recurrent disease and reduce breast cancer specific mortality.

It is hoped that if strong evidence is found in support of lifestyle modifications and better disease outcome, that patients can be given advice about lifestyle and make changes accordingly – however it must be noted that patients do not always adhere to this advice(232). Therefore studies in this area are popular, aiming to define the advice patients should be given to reduce recurrence rates, which is a new clinical challenge due to increasing survival(231).

The evidence for various environmental influences on breast cancer prognosis shall be reviewed to highlight the key modifiable influences on breast cancer outcome. When reading and comparing these papers it is important to note that there is considerable variation in the definition of outcome and also in the time points at which the data is collected. This means that there can be difficulties in making direct comparisons between

papers. For the purpose of this chapter the prognostic outcomes focussed on shall be disease free survival or recurrence, breast cancer specific mortality and all-cause mortality. These are also the outcome measures used in the analysis of survival in a Tayside cohort described in the next chapter. Special focus shall be given to diet, exercise, body weight and hormonal factors, as these are the factors with the biggest body of evidence and that have previously been discussed with regards to environmental risk. As there is a possibility that some prognostic factors may be linked to environmental and genetic risk factors through common biological processes.

As with the literature review of reproductive and environmental risk factors this literature review is journalistic in nature. Medline searches where conducted using "breast neoplasms" AND "prognosis", with an appropriate accompanying MeSH search term (see Appendix 3). Suitable articles were identified using the abstracts with an emphasis on using the larger cohort, case control studies, systematic reviews and meta-analyses. Once again further articles were identified using the references of articles which were identified by the Medline search.

2. Dietary Influence on Prognosis

Diet is of special interest as it potentially plays a role in breast cancer risk, as outlined in the previous chapter. It can also be easily altered to fit recommendations in individuals who are motivated enough. However these studies must always be interpreted with some caution as assessing and quantifying a diet poses considerable challenges. There is also some discrepancy as to when the information on diet is collected – pre-diagnosis, at diagnosis or post diagnosis(230,232). Diet is also expected to change throughout this period and that using self-reported food frequency questionnaires (FFQ) may not provide accurate data. Even in cohort studies which look at participants prior to diagnosis do not take into account prior diet and have the difficulty of how often to assess diet.

The challenges of this type of study are reflected in the inconsistency of the evidence as to whether diet has any influence on prognostic end points, which are usually defined as breast cancer specific mortality, recurrence or disease free survival. Some specific food groups have been studied in particular due to their mechanistic plausibility and their relationship with risk - particularly fruit, vegetables, fibre and fat content.

The HEAL (healthy eating, activity and lifestyle) cohort consisted of 688 women with a median of 6.7years follow up. In this study they used a FFQ to assess fibre intake and carbohydrate intake which were compared with breast cancer specific mortality and recurrence. They found a non-significant inverse relationship between high fibre intake and mortality(233). The hazard ratios (HR) reported between the high and low fibre intake were 0.53 (95% CI 0.23 – 1.23) and 0.75 (95% CI 0.27-1.70). A similar non-significant trend was seen with recurrence (HR = 0.68 (95% CI 0.27 – 1.70)(233).

Fibre is thought to potentially have a protective effect through its ability to decrease oestrogen levels(233) and the levels of inflammatory markers such as C reactive proteins (CRP)(234). Raised levels of these markers have been associated with poor outcomes(235).

The results from McEligot et al associated diets high in fibre, fruit and vegetables with a better outcome in a group of women with breast cancer. In this study diet was assessed by a FFQ for the year prior to diagnosis, which was completed by subjects(236). The patients with the highest versus lowest fibre intake had a HR = 0.48 (95% CI 0.27-0.86; p trend = 0.01). This study also investigated fat, fruit and vegetable consumption. High fruit and vegetable intake was associated with small protective effect against all-cause mortality in women diagnosed with breast cancer; highest versus lowest intake of vegetables HR = 0.57 (95% CI 0.38-0.94; p trend = 0.02) and fruit HR = 0.63 (95% CI 0.38-1.05; p trend = 0.08). Pierce et al in the WHEL study showed a similar but insignificant trend for improved outcome with high fruit and vegetable consumption(237).

The WHEL study followed a cohort of 3088 women with breast cancer, 1537 of these women were placed in an intervention group. These women were given extensive dietary advice and cooking classes with the aim of exceeding their "5 a day". The two groups were then compared in terms of diet and outcomes. The intervention group ate significantly more fruit and vegetables over the 7.3 years follow up ($p<0.001$). However there was no difference between recurrence (HR = 0.96; 95% CI 0.08-1.14, p = 0.63) or overall survival (HR = 0.91; 95% CI 0.72 – 1.15, p = 0.43)(237).

A similar interventional study was carried out looking at dietary fat reduction and breast cancer free survival in women diagnosed with early stage breast cancer. The women's intervention nutrition study (WINS) recruited 2437 women aged between 48 – 79 years up to one year after diagnosis(238,239). These women were then divided into the intervention and control groups; the intervention group then received intensive counselling from a dietician with the aim of reducing dietary fat to 15% of total energy intake. After an average of 60 months of follow up it was found that there was marginal increase in disease free survival in the intervention group – HR = 0.76 (95% CI 0.60 – 0.98; p = 0.077)[12]. The evidence for dietary intervention is not consistent; however there is evidence that a healthy diet with low levels of fat, high fibre, fruit and vegetables is generally good for our health and wellbeing.

It is a similar picture for alcohol consumption, which is an established risk factor for developing breast cancer, as demonstrated in the previous chapter. A number of studies on the topic of outcome and alcohol have produced non-significant results; however some do perhaps suggest a trend for increased mortality with higher alcohol consumption. Holm et al conducted a cohort study in a large group of Danish women with the aim of assessing pre-diagnostic alcohol consumption and breast cancer prognosis. Women who drank the most (>2units a day), when compared to those who drank the least (<1 unit daily), had

small significant increase in risk of breast cancer recurrence - HR = 1.65 (95% CI 1.02 – 2.67; p = 0.04)(240).

In contrast a study of 3088 women that assessed alcohol intake via the Arizona FFQ and 24 hour recall phone calls, concluded that there was no significant association between light alcohol intake (10-290g alcohol per month) and breast cancer recurrence – HR = 0.91 (95% CI 0.71 – 1.18). They also concluded that the highest alcohol consumption (>300g per month) was protective against all-cause mortality when compared to the lowest consumption group – HR = 0.70 (95% CI 0.48 - 1.02)(241). These results are in agreement with Reding et al who also found that alcohol consumption, regardless of quantity, was associated with increased survival: HR = 0.7 (95% CI 0.5 – 0.9)(242).

Patterson et al reviewed all the relevant papers on the subject that had been published up to 2010. The results of some of these are summarised in *Figure 3.1*, illustrating the HR for all-cause mortality and alcohol consumption in women diagnosed with breast cancer. They concluded that there was a lack of consistency in the work on breast cancer outcome and alcohol consumption which warranted more work(231). However, as for diet, there are difficulties with assessing alcohol intake and variable intake over a lifetime; therefore more work is needed to develop conclusive evidence(232,240).

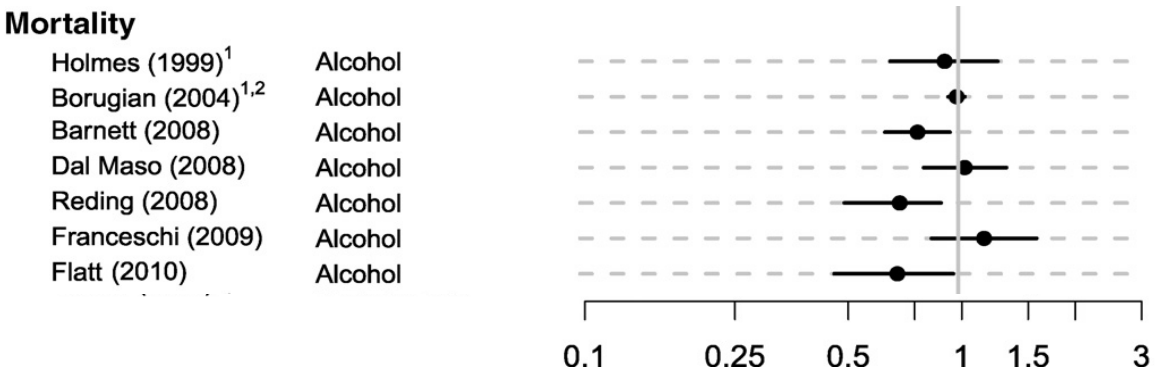


Figure 3.1 – A Forrest plot which demonstrates that there is little consensus as to whether alcohol consumption is associated with mortality in women diagnosed with breast cancer. Adapted from Patterson et al(231)

3. Body Weight and BMI

Having a high body mass index (BMI) has been shown to be a risk factor for the development of breast cancer in postmenopausal but not premenopausal women, as outlined in second chapter of this thesis. It has therefore been hypothesised that by similar mechanisms being overweight may be associated with poorer outcomes in patients diagnosed with breast cancer; namely the increased circulating free oestrogens due to the conversion ability of adipose tissue or the association with increased insulin, insulin resistance and circulating insulin-like growth factor(243–245). It is also important to note that women who are overweight often present with larger and/or higher grade tumours at diagnosis, which are both established poor prognostic indicators(246).

The majority of the evidence suggests that a high BMI or obesity are negative prognostic indicators(244,246–251). However a small number of studies have found no association, for example Carmichael et al failed to find any association in their cohort of 1579 women(249). This study is however smaller in size than the majority of the other studies included in this review, and therefore may not have the statistical power to find a very small but significant association between the two.

A review by Chlebowski and McTiernan in 2002 stated that there had been 34 studies to date on the topic which met their inclusion criteria, and of these 26 produced statistically significant results in favour of a relationship between obesity and poor outcome(252). This is supported by a meta-analysis of 12 papers in 2001 (*Figure 3.2*), which gave an overall HR of 1.56 (95% CI; 1.22-2.0) of highest versus lowest measures of weight(251).

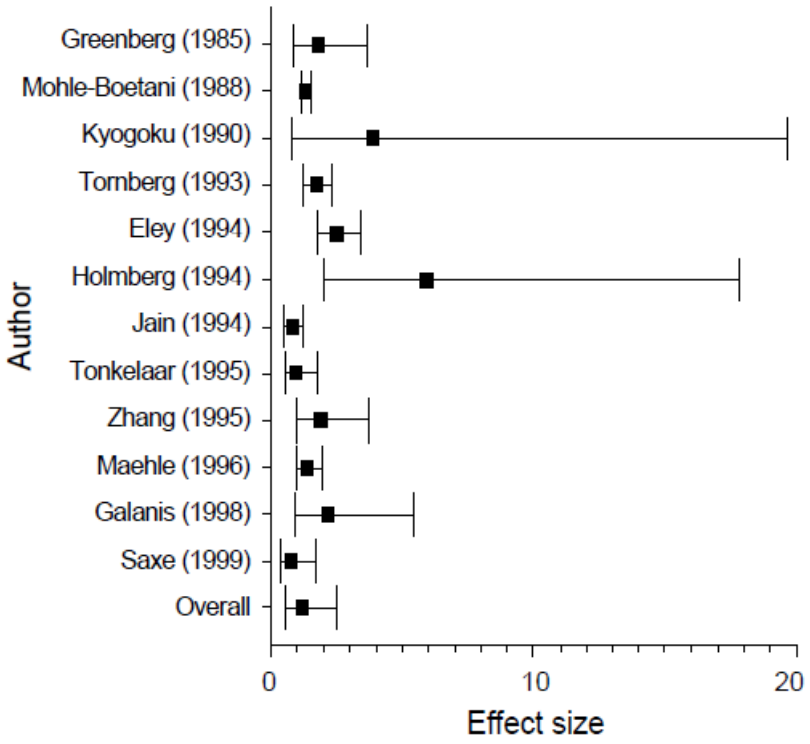


Figure 3.2 - Effect sizes, overall effect size and 95% confidence intervals for BMI and prognosis (all cause death) from the meta-analysis by Ryu et al summarising the findings. Adapted from Ryu et al (251).

More recent data also supports this meta-analysis. For example a study of 5042 breast cancer patients over a, relatively short, average follow up of 3.8 years found HR = 1.44 (95% CI; 1.02-2.03) when comparing women with a BMI >30 to those with a BMI 18.5-24.9(248). However when comparing women who were overweight (BMI 25.0 – 29.9) to those with a normal BMI (18.5 – 24.9) HR = 1.06 (95% CI 0.86 – 1.31) no significant difference was seen. Both these hazard ratios relate to the recurrence/disease specific mortality outcome of this study, as obesity is associated with a number of comorbidities which could potentially increase all-cause mortality rates in women with the highest BMI's(248).

Ewertz et al also found that obesity was an independent prognostic factor, associated with poorer outcomes in women with breast cancer(246). Women with BMI's over 30 were 24.3% more likely to be found to have distant metastases. This same group of women

were also 57.2% more likely to die after their breast cancer diagnosis over an 11.4 year follow up. The authors of this paper questioned whether perhaps the poor prognosis of these women was linked to a decreased effectiveness of adjuvant treatments in women who are overweight(246). This theory though logical still requires the backing of evidence.

The majority of the above studies are in populations of predominately postmenopausal women, and relatively few focus on premenopausal women despite menopausal status influencing if BMI is a risk factor for breast cancer. One paper by Enger et al observed a non-significant trend towards decreased risk of breast cancer death in the women with the highest versus lowest BMI in a group of premenopausal women (253). Finding a 25% reduction in breast cancer death among the women with the highest BMI compared to a normal BMI (p trend = 0.21). However this was a fairly small cohort of 717 women and the result was insignificant, so this being a result of chance cannot be ruled out.

A second paper by Loi et al had a study population that consisted of 74% premenopausal women. In the group of premenopausal women the HR for disease recurrence was 1.50 (95% CI 1.00 -2.26; p =0.06) in obese (BMI >30) in comparison to non-obese women(254). It is not as clear as to whether obesity effects the prognosis in premenopausal women – of the 10 papers referenced by Loi et al only four produced a statistically significant association between the two(254). More work is needed in this are to define the relationship between menopausal status and weight in terms of outcome.

Another area of interest is weight change after diagnosis and its effect on survival or recurrence of breast cancer. This is of special interest as it is very common for women undergoing breast cancer treatment to gain weight (252,255,256). Potential reasons for this weight gain are increased intake, decreased activity or reduced metabolism – the actual mechanism underlying this observed weight gain remains unknown(252).

Caan et al found no association between weight gain and recurrence over an average of 6.1 years follow up(256). In this case recurrence was defined as all local and distant recurrences or development of a contralateral breast cancer. The hazard ratios were 0.8 (95% CI; 0.6 – 1.1) and 1.0 (95% CI; 0.7 - 1.3) in women who gained 5-10% and >10% of their body weight respectively(256).

A study carried out on 5204 women from the Nurses Health Study (NHS) observed a decrease in survival in those who gained weight post diagnosis, a relationship which seemed to be more significant in never smokers (*Table 3.1*)(244). In this study weight gain was associated with both increased risk of recurrence and mortality.

Breast Cancer Deaths	**Category BMI Change (kg/m^2)**				
	> 0.5 Loss	*Maintained*	*0.5 to < 2.0 Gain*	*> 2.0 Gain*	
Never smokers:					
RR (95% CI)	1.01 (0.65 – 1.58)	1.00	1.35 (0.93 – 1.95)	1.53 (1.04 – 2.24)	P trend = 0.03
Past & Current Smokers:					
RR (95% CI)	1.18 (0.85 – 1.63)	1.00	1.10 (0.83 – 1.47)	1.05 (0.78 – 1.43)	P trend = 0.84

Table 3.1 - Summary of the breast cancer specific mortality from NHS stratified by smoking status. Adapted from Kroenke et al(244).

The evidence generally supports the association between BMI and poor outcome in postmenopausal women, but it is less clear if the same relationship exists in premenopausal women. This is in part due to fewer studies having been carried out in populations of premenopausal women. There are indications that weight gain after diagnosis has a negative influence on prognosis. However further studies in this area will need to be carried out to come to a clear consensus as to the importance of weight maintenance after breast cancer diagnosis. It is also unclear as to whether encouraging weight loss in overweight women will improve their outcome. There is a need for more intervention studies to bring clarity as to whether weight loss would be beneficial(257).

There is less published work that tackles the issue of underweight women (BMI <18.5 kg/m^2) and breast cancer prognosis. However it is thought that the relationship between weight and breast cancer survival may be U or J shaped; therefore there is a need to address the survival of women with low BMIs at diagnosis(illustrated in *Figure 3.3*)(258). A large pooling project with almost 15000 participants found that after 7.8 years follow up underweight women (BMI <18.5 kg/m^2) had higher overall mortality – HR = 1.59 (95% CI: 1.18 – 2.13). However they did not find an association with recurrence or breast cancer specific mortality and a low BMI at diagnosis(258). On the other hand a large Korean study found an association between underweight patients and breast cancer specific mortality (HR = 1.49; 95% CI: 1.15-1.93)(259). There clearly needs to be more work done to elucidate the relationship between breast cancer prognosis and low BMI as there are strong indications that it is also a negative prognostic marker.

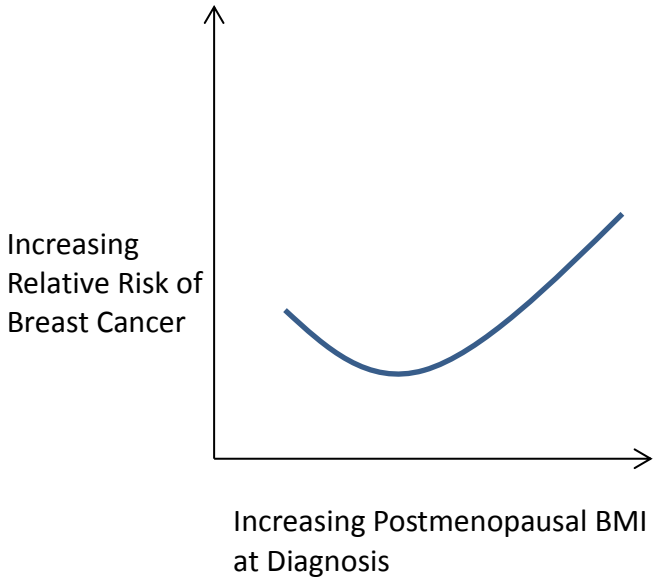


Figure 3.3 - This illustrates the J or U shaped relationship which is thought to exist between breast cancer outcome and BMI in postmenopausal women.

The evidence suggests that obesity and perhaps low body weight has an impact on the outcome of the disease, indicating that both high and low BMI's at diagnosis may have a negative impact on outcome. Additionally weight change during treatment may also have

some influence over a patient's prognosis. However the mechanism underlying these relationships is unknown and if this relates to circulating oestrogen levels is still an area of active research.

4. Physical Activity

Physical activity is associated with decreased breast cancer risk, but the relationship between physical activity and breast cancer prognosis needs further elucidation(260). The difficulty in assessing physical activity is selecting the time point at which to assess activity; for example is it best to assess exercise pre-diagnosis, post diagnosis, at another specific time point or estimate lifetime exercise. A clear decision as to which one of these is the best or most relevant time period has not been agreed upon, making it more difficult to directly compare the evidence from the various published papers.

A recent systematic review of the role of physical activity on cancer prognosis in general found that the majority of the evidence supported a relationship between increased exercise and increased breast cancer survival(261). It summarised that of the 17 relevant observational studies identified in their database search 4 papers produced null results, 7 found a non-significant associations and the remaining 6 identified a significant association between increased physical activity and increased survival. However publication bias cannot be ruled out and these published papers with null results may represent the tip of the iceberg. There is no way of knowing if this bias is affecting this or any of the factors discussed in this chapter.

A study which specifically examined the impact of pre-diagnostic physical activity on breast cancer outcomes, found that women who had partaken in moderate lifetime exercise were less likely to die from breast cancer than those who had done no exercise (HR=0.64; 95% CI=0.43-0.93)(262). It also found that this relationship was strengthened in those who had done exercise during menopause. The caveat with this paper is that it required self-

reported estimates of exercise at various points in life, which have the potential to be inaccurate.

Other papers have assessed exercise following diagnosis at various time points, for example 6, 18 and 36 months after diagnosis. Chen et al found that at 36 months regular exercisers versus non exercisers had a lower risk of recurrence and disease specific mortality; HR = 0.6 (95% CI; 0.47 – 0.76)(263). However the authors highlighted that low levels of exercise may be due to poor health, which in turn is responsible for the increased survival in those who are doing more exercise.

Potential mechanisms that may underlie the observed increase in survival and decreased recurrence rates with increased physical activity include: decreased oestrogen levels, increased immune functions, lower body fat or decreased insulin resistance(260,262). The relationship between physical activity and breast cancer prognosis is hard to interpret. More work may be required to identify when in particular exercise is important in terms of survival – lifelong, after menopause or after diagnosis? Additionally as highlighted by Chen et al post-diagnosis exercise is in part dependent on the patients' health and response to treatment, therefore the patients partaking in the highest levels of exercise are in better health than those who do not.

5 Hormone Replacement Therapy and Survival

Hormone replacement therapy (HRT) is generally well accepted as a risk factor for developing breast cancer due mainly to three large studies which were published in the 1990's (117,118,178). These papers have been discussed in depth during the course of the previous chapter. However the effect of HRT on breast cancer prognosis has been a recent area of interest. It has been noted by numerous papers that there seems to be increased survival in women using HRT at the time of diagnosis. The reasons for these observations are still under debate – it may be due in part to the increased mammographic surveillance

these women are under as they have a higher breast cancer risk. There is also some evidence that HRT is associated with tumours that have better prognostic factors(264–268). For example current long term use of HRT was associated with the diagnosis of a grade 1 opposed to a grade 3 tumour when compared to women who never used HRT (OR = 0.3; 95% CI 0.2 – 0.5)(264). This may again be due to mammographic surveillance or greater awareness of breast cancer and therefore self-examination in this population of women.

A paper published in 2008 which followed a group of 2660 breast cancer patients for approximately 9.3 years, found that women who were using HRT at diagnosis were at a lower risk of dying due to the breast cancer (HR = 0.57; 95% CI 0.41 – 0.79). However, this paper found no association between survival and past use(264). This is in agreement with Barnett et al who found a decreased risk of death in women who used HRT for >4years than non-users (HR = 0.65; 95% CI, 0.51 to 0.84; P= 0.001). The authors also found no association between prognosis and age at menopause/menarche, smoking history or previous use of the oral contraceptive pill (OCP)(266).

Other authors have also specifically found that women who use HRT have tumours that are more likely to be smaller, lower grade and node negative; which are some of the most important prognostic indicators in breast cancer care(267). Fletcher et al expanded on this idea by adjusting for these characteristics in their statistical analysis. Prior to adjusting for tumour characteristics HRT use was associated with increased survival (HR = 0.64; 95% CI 0.41 – 1.00). This modest increased in survival was lost after adjusting for the tumour characteristics, suggesting that these tumour characteristics may in part be responsible for the survival benefit of taking HRT(268).

The explanation of the better prognosis associated with HRT use is still poorly understood, however there have been several suggestions made as to why this is the case. These

include the tumour characteristics, socioeconomic status, educational level of women taking HRT, screening, or that these women may be more likely to be health conscious(268). Additionally HRT is thought to increase risk as it increases the amount of available exogenous hormones, when a patient it diagnosed with breast cancer this exogenous source of hormone will be removed which may also influence prognosis.

The relationship between the OCP and breast cancer prognosis is less clear, with the majority of the evidence finding no association(266,269–271). This lack of association is consistent regardless of duration(271), latency or recency of use(270). The body of the evidence suggests that there is no association between OCP use and breast cancer prognosis. However there is more evidence needed to define the relationship between HRT or HBC use and breast cancer survival; as current indications suggest current HRT use has a survival benefit, but HBC use does not.

6. Socioeconomic Status

A high socioeconomic status (SES) has been established as a risk factor for developing breast cancer(272), however like many other cancers a lower SES has been linked to poor prognosis(256,272–278). Various reasons for this link have been hypothesised ranging from access to health care, treatment options and tumour biology(272,273,279).

Making direct comparisons between papers can be difficult due to the various different measures of SES – for example one paper used the occupation as a measure of deprivation(272), whereas other papers have used the Carstairs deprivation index score(276). However it must be noted that regardless of what measure of SES used the results remain generally constant with lower SES being associated with a worse prognosis than the highest SES.

A Swiss study on a group of 3920 women diagnosed with breast cancer before the age of 70 used occupation to determine SES. This meant that housewives were classified as being of a low SES regardless of their home situation, which may prove to be inaccurate. Perhaps an estimated household income or the area in which they lived would have been a more accurate way of estimating SES in this study. However the results of this study are in agreement with the bulk of the literature finding that women of the lowest SES were at the highest risk of dying from breast cancer – HR = 2.4 (95% CI 1.6 – 3.5)(272). This hazard ratio only partially decreased (HR = 1.8; 95% CI 1.2 – 2.6) when the results were adjusted for tumour characteristics, delayed diagnosis and treatment options - leading the authors to conclude that SES is an independent prognostic factor(272).

There have also been papers published that specifically focus on the Scottish population, which is particularly relevant to survival analysis which has been carried out on a Tayside cohort in the following chapter. Thomson et al found in their data set of 21,751 women diagnosed with breast cancer before the age of 85, that there was an 8.7% survival difference between the highest and lowest deprivation categories(275). A second paper which looked at women diagnosed with breast cancer from 1986 to 2000 in the Scotland used the Carstairs deprivation index score to categorise SES. It found that in women diagnosed with breast cancer between 1996 to 2000 there was a 4.1% difference in 5 year survival between women in the highest and lowest SES groups(276).

A large study conducted in the Netherlands also agreed with the above results. Finding a significantly lower 10 year overall survival in women in the very low SES group in comparison to the very high SES group (HR = 1.10; 95% CI 1.06 – 1.13; $p < 0.001$)(277). The results of this paper are summarised in *Figure 3.4*.

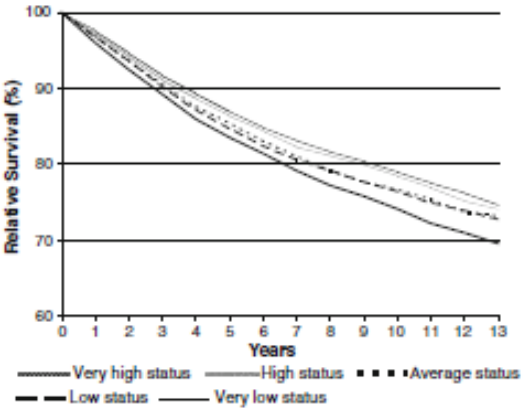


Figure 3.4 - A graphic representation of the relative survival of women diagnosed with breast cancer according to their assigned SES. Adapted from Bastiaannet et al (277).

It is clear from the evidence that SES does have an association with patient prognosis. However what this is due to is still unclear – there is evidence that in part it is related to tumour biology for example one paper examined the presence of p53 mutations in deprived patients(273), and other tumour size(277). It could also be related to assess to health care and to the treatment options available to patients, further work will need to be carried out to elucidate the mechanism behind this association(279). This subject is covered in more depth in a further chapter with regards to the survival analysis carried out on a Tayside cohort, which plays particular attention to the effect that deprivation has on breast cancer survival.

7. Conclusion

There is a great deal less epidemiological work on the association between various modifiable lifestyle factors and breast cancer prognosis in comparison to the work done on breast cancer risk. The value in this work lies in the advice that can be offered to patients after diagnosis, which could allow them to make lifestyle changes to improve their outcome(232).

The greatest evidence is in support of an association between improved prognosis and a normal BMI at diagnosis and, weight loss after diagnosis. The literature also indicates that

the use of HRT at the time of diagnosis and a higher SES may be prognostically favourable. However the evidence is less clear for the role of diet, exercise or the use of the OCP. One reason for this may be the difficulties in studying these factors due to the difficulties in setting parameters, relying on short follow up and self-reporting.

However, some of these factors may influence both prognosis and risk but in different ways. An example of this is SES as a high SES increases the risk of breast cancer but is associated with a better outcome than a lower SES. The reason for this difference is once again not known, but it may relate to gene-environment interactions or may relate more to behavioural reasons. The study of gene-environment interactions and breast cancer prognosis is in the extremely early stages. Preliminary data suggests that it is likely to be different low penetrance polymorphisms which influence prognosis and risk(280). To date there is no reliable evidence that any of the SNPs which predispose a woman to breast cancer have any influence on prognosis, with only one exception which is the SNP located at 8q24(280) - first identified by Easton et al(96). There has been a small amount of work which looked at the relationship between polymorphisms encoding interleukins and breast cancer survival. This same study also looked at the relationship between a number of these polymorphisms and environmental prognostic factors to look for any possible interactions(281). They did find several significant interactions between two SNPs in IL23R and a lower Native American ancestry and breast cancer specific survival. However, this study was conducted in a largely African-American and Hispanic population who have already been shown to have a poorer outcome than Caucasian women. Additionally the majority of breast cancer data is based on Caucasian populations. The lack of data in this area may be down to a multitude of factors, including the lack of knowledge into the genetics underlying prognosis, lack of current research in the field and publication bias. The role of gene-environment interactions in breast cancer outcome is poorly understood at this moment of time and further work is required in this area. Once again future

research may provide a greater understanding of disease and may add to the prediction of patient prognosis.

From the literature there is an argument that, with the exception of prior HRT use, sensible advice on healthy lifestyle choices including a healthy diet, exercising and keeping within the normal BMI range is the best advice that can be offered women diagnosed with breast cancer to improve their outcomes. There are once again indications that the environmental factors which affect risk also play a role in prognosis. For example a high post-menopausal BMI is associated with an increased risk and a poorer prognosis; the reason for this may be that the increased circulating oestrogen increases risk and results in a poorer outcome. Therefore once again it could be postulated that ESR1 and FGFR2 polymorphisms may have a negative effect on prognosis too or gene-environment interactions may also influence prognosis. This is a potentially interesting future field of research which could also result in elucidation of pathogenic mechanisms and novel therapeutic breakthroughs.

Breast Cancer Survival and Deprivation

1. Introduction

As mentioned in the previous chapter the incidence of breast cancer is increasing worldwide(1); it is because of this high incidence of disease that there has been such extensive work carried out to assess various risk and prognostic factors which contribute to its development. For example women from the most deprived areas (or low SES) have been found to have a poorer outcome than those from more affluent areas (or SES). However the reason for this disparity between those in high and low socioeconomic groups remains unknown – it has been suggested it may relate to later presentation, lower awareness of the disease, poor access to treatment or biological factors(273,282–284).

One such large scale study investigating cancer survival throughout Europe is the EUROCARE project, which has recently published its most up to date results in EUROCARE-5(285). The most recent results found the European mean 5 year survival for breast cancer to be 81.8% (95% CI; 81.6% - 82.0%). This was unfortunately considerably lower in the UK and Ireland at 79.2% (95% CI; 79.0%-79.4%) 5 year survival(285). There are various possible reasons for the lower survival in the UK including population health, access to care, screening, social and lifestyle factors. However, when looking at this type of data it is always important to approach the results with a certain level of caution as there are many incomplete cancer registries in some of the major Europeans countries – including France, Germany and Italy. The health of the population within in these blank spots is unknown, which may result in an over or under estimation of survival in that country. In addition, EUROCARE fails to take into account other sociodemographic and environmental factors which have been demonstrated by the evidence in the previous chapter to influence outcomes(286).

In light of the proposed association between low SES and lower mean survival, it was of interest to assess survival in a smaller Scottish cohort who had all been diagnosed in Tayside between 2000 and 2004. The aim of this survival analysis was to look at survival as a whole in Tayside to allow comparison with large studies like EUROCARE-5 and smaller studies which have investigated survival in other populations. In addition, it aims to investigate the impact of key prognostic factors on survival, including some of those previously mentioned. These prognostic factors include deprivation, tumour stage at diagnosis, oestrogen receptor status (ER), progesterone receptor status (PR), human epidermal growth factor (HER2) receptor status and age. Socioeconomic status was the only environmental risk or prognostic factor which was taken into account when this dataset was collected. Therefore this unfortunately means that analysis of the other environmental and genetic factors discussed in the previous chapters, such as BMI, HRT or HBC use, cannot be assessed in this analysis; however this is something which would be of interest to pursue in this future.

2. Method of Analysis

The data from 1851 women diagnosed with primary breast cancer in Tayside was collected between 2000 and 2004. Anonymised information was collected on date of diagnosis, patient age at diagnosis, grade, tumour size, lymph node status, ER, PR and HER2 status, treatment, specific subtype of cancer and the postcode at time of diagnosis. The pathological parameters were collected and recorded by a single team of pathologists based in Ninewells hospital. All the patients were managed by the same multidisciplinary team. These patients were then followed up until December 2013 with information on survival collected from medical records and death certificates. For those participants known to have died the cause of death was obtained from the death certificates. Outcome

was recorded as alive, recurrence and date of this, or date of death with the specific cause of death also recorded.

The data was then analysed using SPSS(287), which allowed the calculation of life tables, Kaplan-Meier plots and Cox regression analysis for all the various factors included in analysis. Kaplan-Meier analysis and Life tables where used to calculate 5 and 10 year cumulative survival rates, quoted with 95% confidence intervals (CI) and standard errors (SE) respectively. For further information on risk cox regression analysis was used to calculate hazard ratios (HR) quoted with 95% CI, which also allowed various co-variants to be included in analysis.

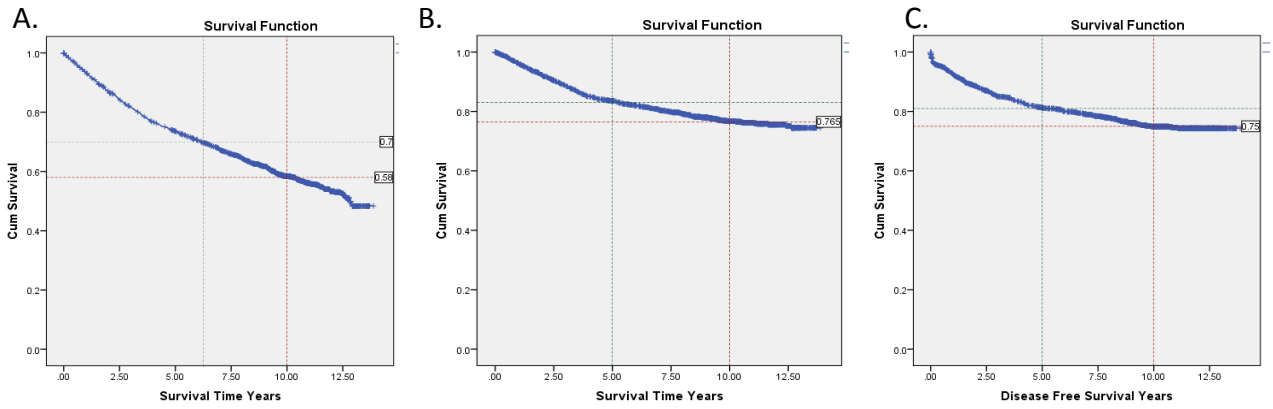
Three outcomes were used for each analysis All-Cause Mortality (death by any cause including breast cancer), Breast Cancer Specific Mortality (the cause of death was confirmed by the death certificate) and Disease Free Survival (recurrence was recorded in medical records). Each outcome was analysed to determine if any of the prognostic factors influenced it; these included age, deprivation, tumour size (mm), invasive grade, nodal status, tumour type and ER, PR, and HER2 status.

Age was divided into three age categories with reference to the screening program - groups <50 years (pre-screening), 50-69 years (screening) and ≥70 years (post-screening) – for data analysis. Tumour size was also analysed with the women divided into groups, based on the T1, 2 and 3 staging system used in Tayside - ≤20mm, 20 - <50mm, ≥50mm.

Finally, deprivation was classified using the patient's postcode at diagnosis and the 2001 Carstairs index(288). Carstairs classifies postcode areas into 10 deprivation categories dependent on a number of factors including overcrowding, number of cars, male unemployment and social class based on the 2001 census. As the data set was collected in the period around 2001 using this measure of deprivation was deemed the best option, as

it was up to date for this cohort. For this analysis postcodes were divided into 10 deprivation deciles, but due to small numbers of women in some groups the deciles were then grouped into 3 SES categories: affluent -deciles 1 and 2; intermediate - deciles 3-7; deprived – deciles 8-10. These groups were divided simply to try and divide the women in such a way there were numerically similar group sizes. Additionally, to eliminate any bias caused by the division of the groups in this way the top 3 and bottom 3 groups were compared to look for any survival differences across all outcomes.

i)All Comers



ii)Operable Cancers Only

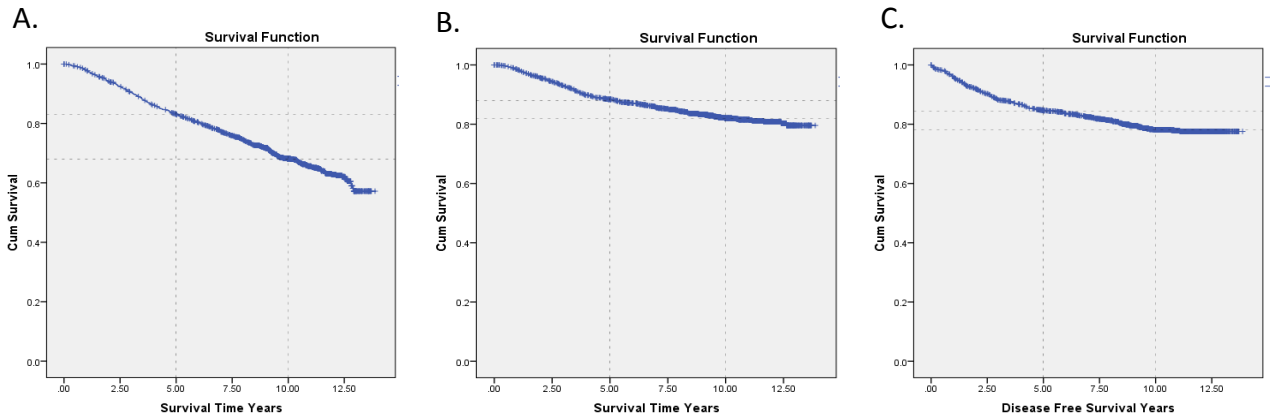


Figure 4.1 Kaplan Meier Plots illustrating how survival over time for each survival outcome for both all cancers and operable cases only i)All Comers (operable and inoperable) and ii) operable cancers only. A) All-Cause mortality; B) Breast Cancer Specific Mortality; C) Disease Free Survival.

3. Results

All 1851 women had a primary breast cancer and were therefore included in the analysis, unless there were missing values for a particular analysis. Over a median follow up period of 8.8 years, which ranged between 0-13.9 years, there were 768 deaths and 342 of these were breast cancer specific deaths identified from death certificates at the national records in Edinburgh. In the same time period of approximately 8 years there were 405 recurrences of breast cancer, identified from medical records.

3.1 Five and Ten Year Survival

Survival was assessed both in all cancers (all invasive and in situ lesions) and operable cancers only, the latter to allow comparison with other studies as they generally only include operable cases. Initially survival in all cancers was assessed for 5 and 10 year survival across all 3 outcomes.

Breast cancer specific survival was higher at both 5 and 10 years than all-cause mortality when all cancers were included in analysis *(Table 4.1 and Figure 4.1)* and when survival was calculated using both life tables and Kaplan-Meier analysis. Five year survival was 82% (+/-1%) for breast cancer specific mortality and was only 70% (+/-1%) for all-cause mortality. These survivals were lowered at 10 years to 76% (+/-1%) for breast specific mortality and 56% (+/-1%) for all-cause mortality.

Disease free survival shows similar cumulative survival rates to those of breast cancer specific mortality; at 5 years it was 80% (+/- 1%) and at 10yrs it was 75% (+/- 1%) for all cancers (*Figure 4.1).* A second analysis was performed excluding the 168 cases of ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) these failed to significantly alter cumulative survival rates, and so it was decided to continue the analysis with them included.

Variable	5 year Cumulative Survival (% +/-SE)			10 year Cumulative Survival (5 +/- SE)		
	All-Cause	*Breast Cancer Specific*	*Disease Free Survival*	*All-Cause*	*Breast Cancer Specific*	*Disease Free Survival*
All Patients	70% (+/-1%)	82% (+/- 1%)	80% (+/- 1%)	56% (+/-1%)	76% (+/- 1%)	75% (+/- 1%)
Operable Patients	80% (+/-1%)	87% (+/-1%)	84% (+/-1%)	66% (+/-1%)	82% (+/-1%)	78% (+/-1%)
Age						
<50yrs	80% (+/- 2%)	82% (+/- 2%)	75% (+/- 2%)	69% (+/- 3%)	75% (+/- 3%)	68% (+/- 3%)
50-69yrs	83% (+/- 1%)	88% (+/- 1%)	85% (+/- 1%)	71% (+/- 2%)	84% (+/- 1%)	80% (+/- 1%)
>70yrs	46% (+/- 2%)	77% (+/- 2%)	76% (+/- 2%)	26% (+/- 2%)	64% (+/- 3%)	69% (+/- 2%)
Deprivation						
Affluent	74% (+/- 2%)	84% (+/- 2%)	81% (+/- 2%)	58% (+/- 2%)	78% (+/- 2%)	76% (+/- 2%)
Moderate	73% (+/- 2%)	85% (+/- 1%)	82% (+/- 2%)	60% (+/- 2%)	78% (+/- 2%)	77% (+/- 2%)
Deprived	63% (+/- 3%)	75% (+/- 2%)	76% (+/- 2%)	49% (+/- 3%)	71% (+/- 3%)	70% (+/- 3%)
Nodes						
Negative	77% (+/- 1%)	89% (+/- 1%)	87% (+/- 1%)	63% (+/- 2%)	84% (+/- 1%)	84% (+/- 1%)
Positive	65% (+/- 2%)	73% (+/- 2%)	67% (+/- 2%)	49% (+/- 2%)	64% (+/- 2%)	58% (+/- 2%)
Invasive Grade						
1	84% (+/- 2%)	96% (+/- 1%)	97% (+/- 1%)	72% (+/- 3%)	92% (+/- 2%)	92% (+/- 2%)
2	74% (+/- 2%)	86% (+/- 1%)	83% (+/- 2%)	58% (+/- 2%)	79% (+/- 2%)	76% (+/- 2%)
3	63% (+/- 2%)	71% (+/- 2%)	67% (+/- 2%)	49% (+/- 2%)	65% (+/- 2%)	62% (+/- 2%)
Size (cm)						
≤2cm	87% (+/- 1%)	94% (+/- 1%)	91% (+/- 1%)	73% (+/- 2%)	89% (+/- 1%)	86% (+/- 1%)
2-5cm	73% (+/- 2%)	80% (+/- 2%)	75% (+/- 2%)	56% (+/- 2%)	73% (+/- 2%)	69% (+/- 2%)
>5cm	58% (+/- 5%)	66% (+/- 5%)	58% (+/- 5%)	41% (+/- 5%)	56% (+/- 5%)	48% (+/- 5%)
ER Status						
Positive	76% (+/- 1%)	87% (+/- 1%)	84% (+/- 1%)	60% (+/- 2%)	80% (+/- 1%)	78% (+/- 1%)
Negative	56% (+/- 3%)	64% (+/- 3%)	59% (+/- 3%)	46% (+/- 3%)	59% (+/- 3%)	57% (+/- 3%)
PR Status						
Positive	78% (+/- 1%)	88% (+/- 1%)	86% (+/- 1%)	61% (+/- 2%)	82% (+/- 1%)	79% (+/- 1%)
Negative	61% (+/- 2%)	71% (+/- 2%)	66% (+/- 2%)	50% (+/- 2%)	65% (+/- 2%)	64% (+/- 2%)
HER2 Status						
Negative	74% (+/- 1%)*	84% (+/- 1%)*	81% (+/- 1%)	57% (+/- 2%)*	77% (+/- 1%)*	74% (+/- 1%)
Positive	67% (+/- 3%)*	77% (+/- 2%)*	72% (+/- 3%)	58% (+/- 3%)*	73% (+/- 3%)*	69% (+/- 3%)

Table 4.1 – Summary of all cumulative 5 and 10 year survival rates calculated for all variables investigated by life tables (* insignificant log rank test).

A second analysis was carried out which included only the 1542 operable breast cancer cases in the cohort to provide 5 and 10 year cumulative survivals which are comparable to other published data. This meant that the 5 and 10 year survival rates were increased when compared to that of all cancers; 5 year survival for all-cause survival increased by 10% to 80% (+/-1%) and breast cancer specific survival to 87% (+/-1%)(*Table 4.1* and *Figure*

4.1ii). This increased survival is to be expected as women with operable cancer by definition have a better prognosis and are generally more medically fit.

The relationship between all-cause mortality and survival is linear in both the all cancers and operable cancers analyses. Whereas the relationship between breast cancer specific cancer mortality and survival is curved indicating that the chance of surviving breast cancer increases as the time from diagnosis increases. The difference between all-cause and breast cancer specific survival was the same in for both all cancers and operable cancers. A similar curved relationship is seen between recurrence and time, which reflects that the highest chance of recurrence is within the first 5 years after diagnosis

3.2 Survival and Year of Diagnosis

The women in this cohort were recruited between 2000 and 2004, and so separate analysis

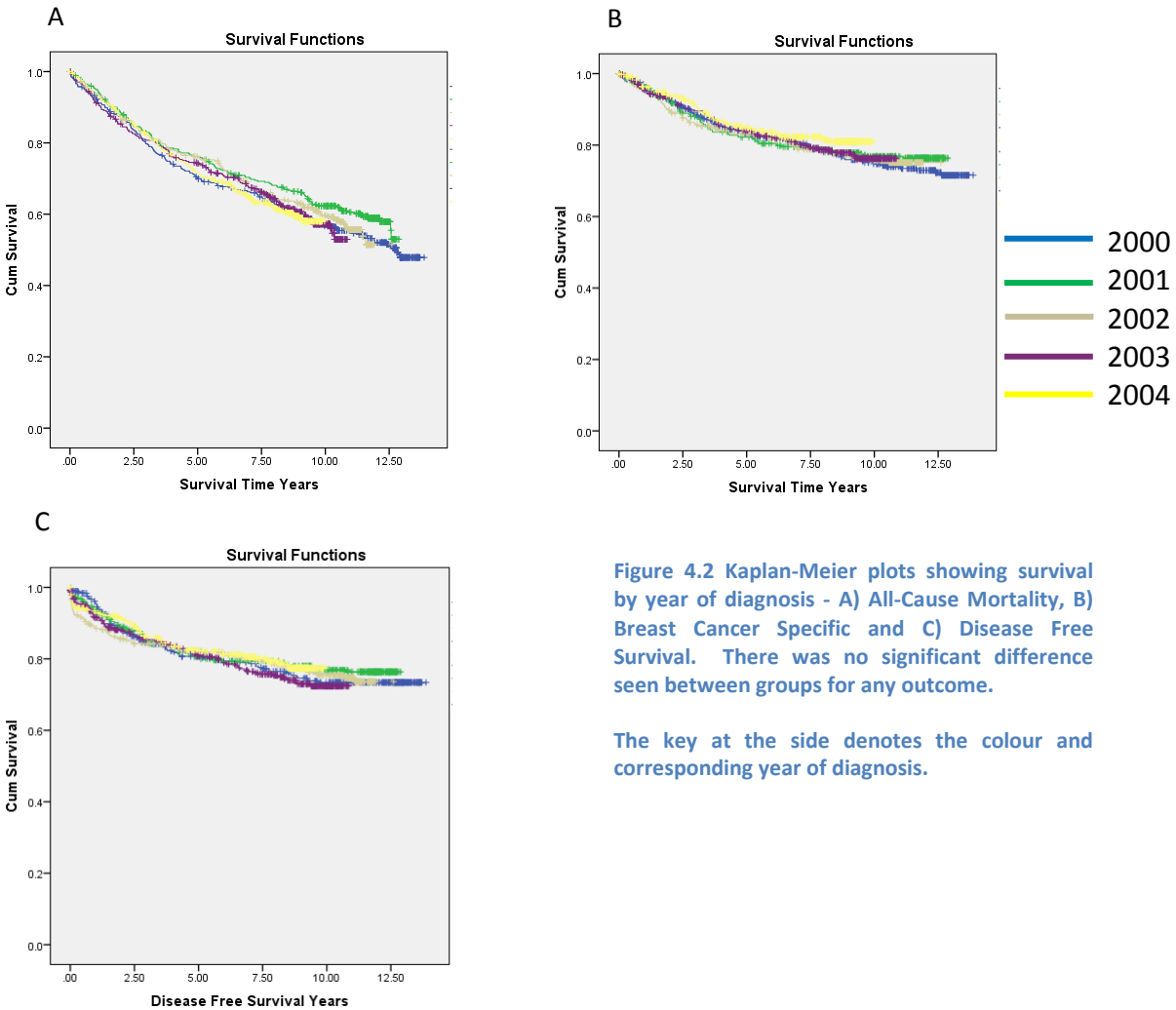


Figure 4.2 Kaplan-Meier plots showing survival by year of diagnosis - A) All-Cause Mortality, B) Breast Cancer Specific and C) Disease Free Survival. There was no significant difference seen between groups for any outcome.

The key at the side denotes the colour and corresponding year of diagnosis.

was carried out to identify any improvement in survival dependent on the year of diagnosis. The hypothesis being that women diagnosed in 2004 have a better outcome than those diagnosed in 2000. The cohort was therefore divided into 5 groups dependent on the year they were diagnosed. This initial Kaplan-Meier analysis failed to show any significant difference between year of diagnosis and survival across all outcomes (*Figure 4.2*).

Therefore, a univariant cox regression analysis was undertaken to look for any associations between the year of diagnosis and survival (*Table 4.2*). This once again failed to identify any significant relationship between the year of diagnosis and any of the three outcomes. However, there was an insignificant trend towards increased survival (p >0.05) between 2000 and 2004 for both breast cancer specific survival and disease free survival (*Table 4.2*). This suggests there may be an improvement in survival over this time, which is not significant due to small numbers and short time period of recruitment used.

	Unadjusted Hazard Ratios (95% CI)	***P value***
All-Cause Mortality		
2004	1.00	0.4
2000	0.97 (0.78-1.20)	
2001	0.82 (0.65-1.03)	
2002	0.92 (0.73-1.16)	
2003	0.98 (0.78-1.23)	
Breast Cancer Specific Mortality		
2004	1.00	0.63
2000	1.28 (0.93-1.77)	
2001	1.16 (0.84-1.61)	
2002	1.24 (0.88-1.73)	
2003	1.19 (0.85-1.65)	
Disease Free Survival		
2004	1.00	0.74
2000	1.11 (0.82-1.51)	
2001	0.997 (0.73-1.37)	
2002	1.10 (0.80-1.52)	
2003	1.20 (0.88-1.62)	

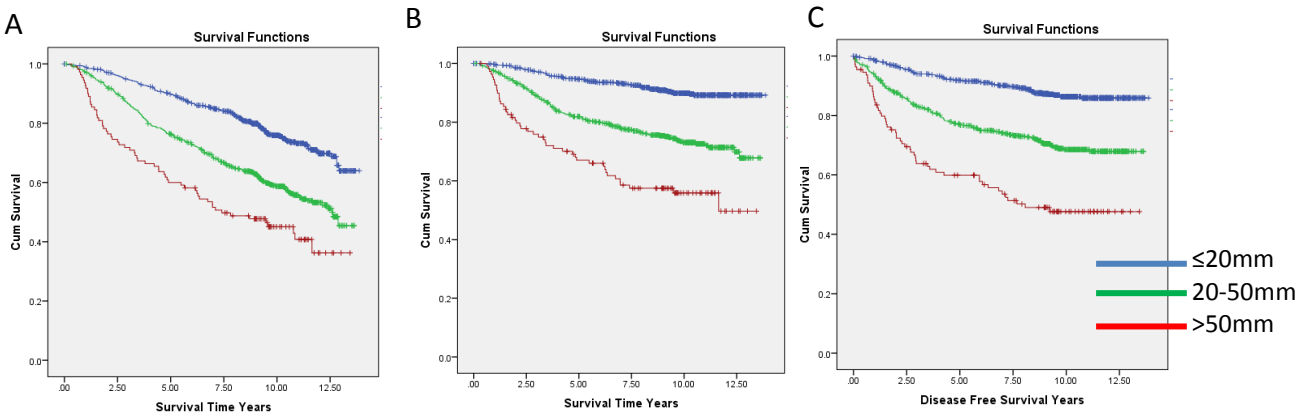
Table 4.2 – Univariant cox regression analysis for each outcome by year of diagnosis. The hazard ratios failed to be significantly different for one another; however there appeared to be a trend for increasing survival between 2000 and 2004 when looking at breast cancer specific mortality.

3.3 Survival, Stage and Receptor Status

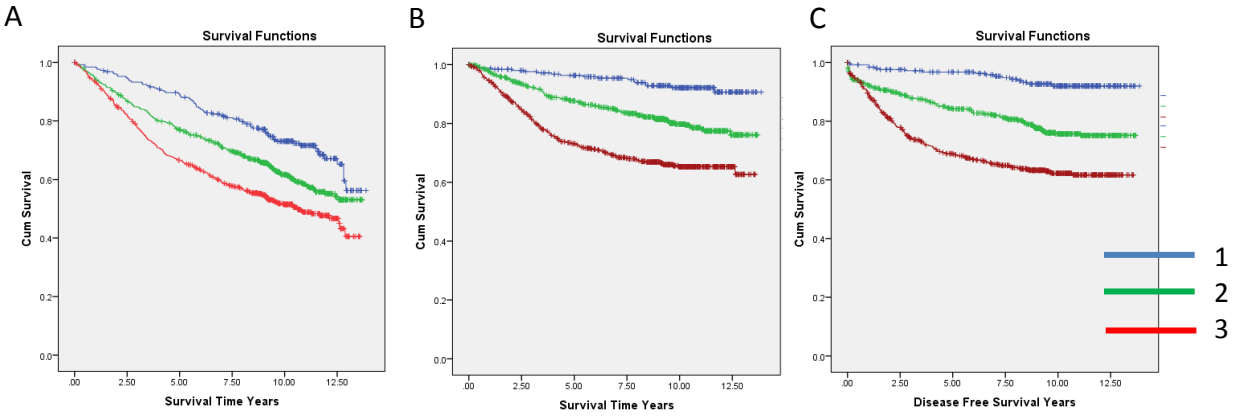
Tumour stage is defined as the size, invasive grade and lymph node status of the tumour; all of these factors are already associated with breast cancer outcome. The influence of these factors was assessed in this cohort to confirm that these factors have the expected relationship with survival. These results do confirm that positive nodal status, large tumours and high invasive grade are all associated with a significant decrease in 5 and 10 year survival for both all-cause mortality and breast cancer specific mortality (log rank test - $p < 0.0001$)(*Table 4.1; Figure 4.3*).

Receptor status has also been previously associated with prognosis, this may be in part due to the fewer number of treatment options available for ER and PR negative tumours. In this cohort ER and PR negative tumours were associated with a lower 5 and 10 year survival for all outcomes (log rank test – $p<0.0001$) (*Figure 4.4i and ii*).

i)Tumour Size



ii) Invasive



iii) Nodal Status

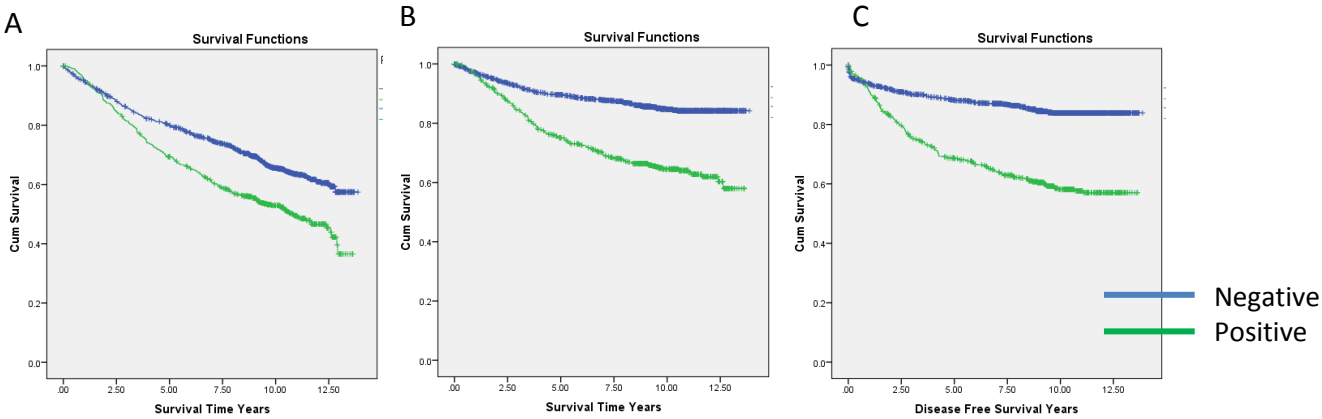
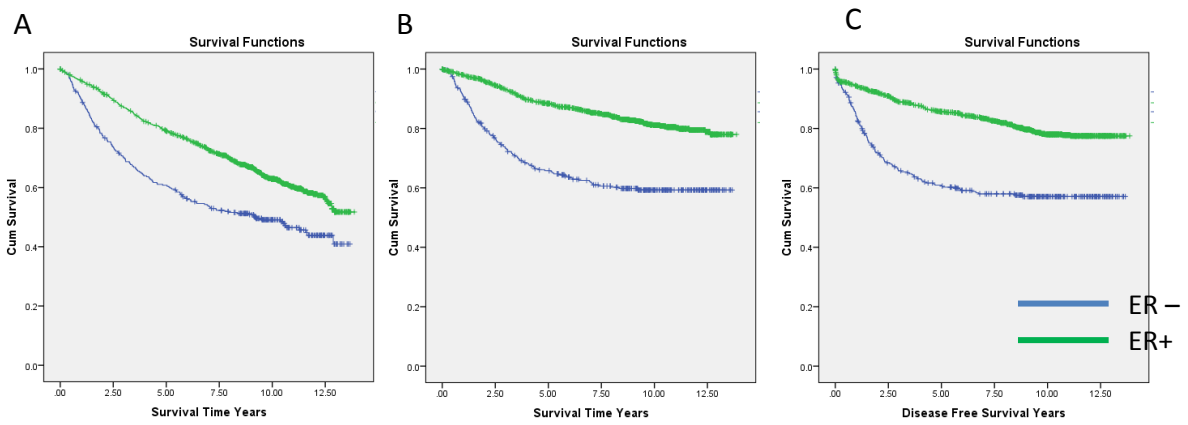


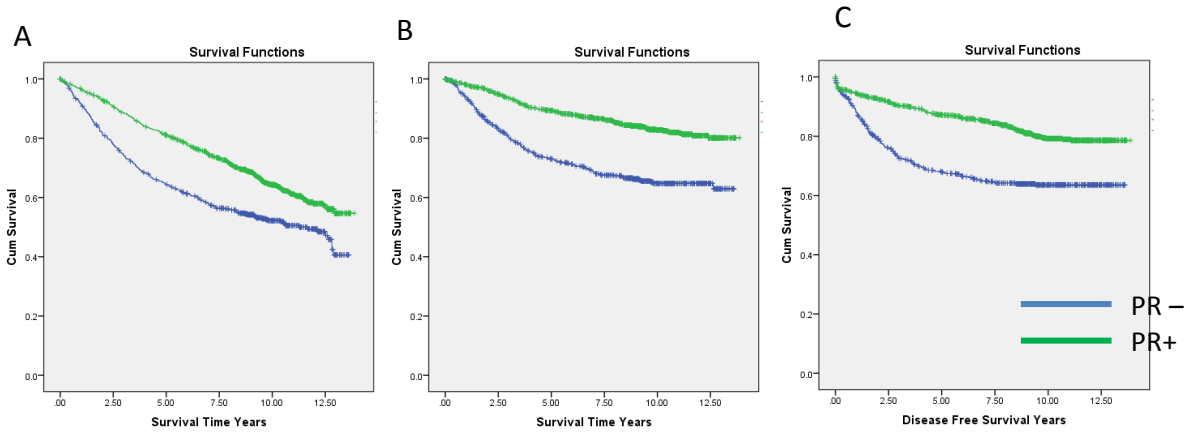
Figure 4.3 - Kaplan-Meier Analysis of survival and tumour size, invasive grade and nodal status for each outcome: A) All-Cause, B) Breast Cancer Specific and C) Disease Free Survival. All the relationships reached significance on the log rank test (p< 0.0001).

At the side of each set of plots the colour and corresponding variable are coded in a key.

i)ER Status



ii) PR Status



iii) HER2 Status

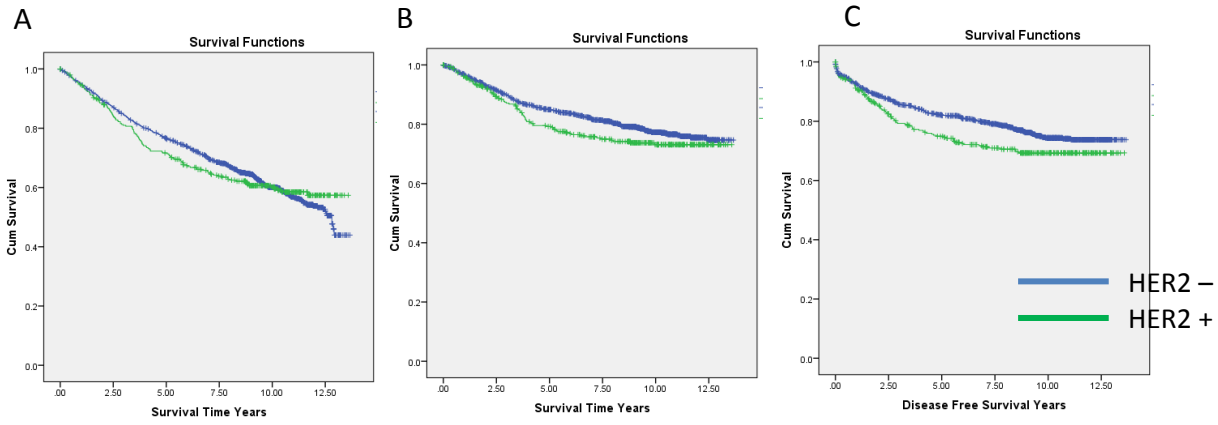


Figure 4.4 - Kaplan-Meier analysis of survival and receptor status for all three outcomes: A) All-Cause mortality, B) Breast Cancer Specific mortality and C) Disease Free Survival. The relationship between ER/PR status (I and ii) and all three outcomes was significant (log rank test), but only the disease free survival outcome had a significant relationship between survival and HER2 status (iii).

At the side of each set of plots the colour and corresponding variable are coded in a key.

On the other hand, there was a no association found between HER2 (human epidermal growth factor receptor) status and all-cause mortality or breast cancer specific mortality

survival outcomes (log rank test p<0.05). However, there was a significant increase in recurrence associated with HER2 positive cancers – 5 year disease free survival in women with HER2 negative cancers was 81% (+/-1%) and for positive cancers was 72% (+/-3%)(*Table 4.1; Figure 4.4iii*).

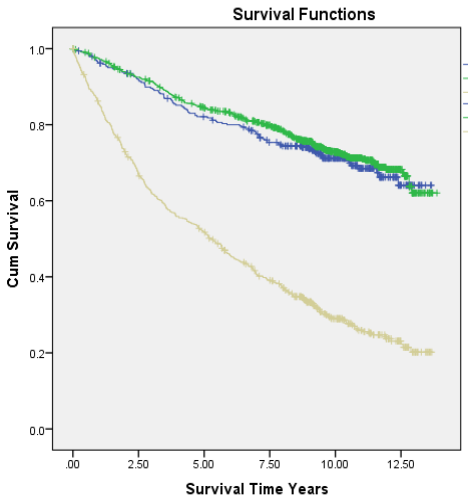
3.4 Age and Survival

Previously, there has been an association made between breast cancer survival and age, which is thought to be for a number of reasons. It was also suggested by the authors of the EUROCARE study that survival in the older UK population may partly explain the difference in 5 year survival between the UK and Europe(285). Therefore, women were divided into groups by age which reflected the UK screening program - <50 years, 50-69 years and >70 years.

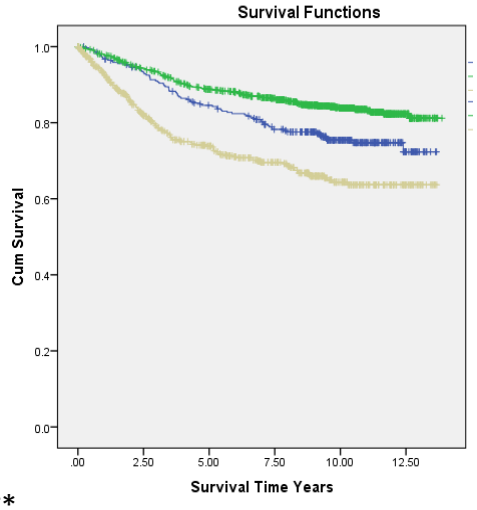
It was found that women in the oldest groups had the lowest all-cause and breast cancer specific survival (*Table 4.1*). The women who were <50 or 50-69 years old had similar all cause survival, but women in the <50 years group have a slightly lower breast cancer specific survival – 5 year survival in those <50 years was 82% (+/-2%) compared to those between 50-69 years which was 88% (+/-1%)(*Figure 4.5*).

The women in the oldest and youngest groups both have worse disease free survival than those of screening age (*Figure 4.5C*): 5yr survival 75%, 85% and 76% for women <50, 50-69 and >70 respectively (p <0.0001). Both the lower breast cancer specific survival and disease free survival in the pre-screening group may represent a number of things, including both more aggressive subtypes of tumour in these women and a later stage of presentation.

A*



B*



C*

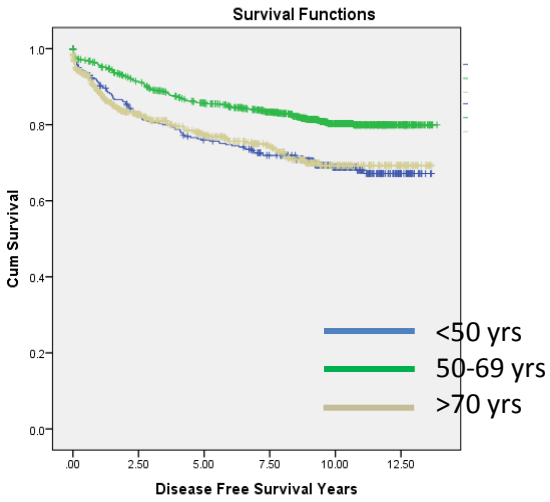


Figure 4.5 - Kaplan-Meier plots depicting the relationship between age and survival, all three relationships were significant (log rank p<0.05). A) all-cause mortality has far lower survival in women over 70 years. B) In the breast cancer specific mortality group the 50-69 year group have the best survival and the >70 years the worst. C) In the disease free survival group both the <50years and >70 years have the highest recurrence rate.

Unadjusted cox regression analysis confirmed these relationships, demonstrating that women in the >70 years groups have the highest risk of both all-cause mortality and breast cancer specific death (*Table 4.3*). Women under 50 years old had 40% decreased risk of breast cancer specific death compared to those over 70 years (p = 0.0002). Women in the screening group had a 61% decreased risk of breast cancer death (p<0.0001), but these values were less than that of all-cause mortality – 50-69 years HR of 0.25 (95% CI; 0.22-0.30) and <50 years HR of 0.28 (95% CI; 0.22-3.4) when compared to >70 years. When disease free survival was examined in the same way women in both >70 year and <50 year old groups have an increased risk of recurrence; but women in the screening group have a decreased

risk (HR = 0.57 (95% CI; 0.46-0.72; <0.0001)(*Table 4.3*).

A multivariant cox regression model was then used to analyse if deprivation, tumour size, invasive grade or nodal status had confounded these results (*Table 4.3*). The relationship between age and all-cause and breast cancer specific mortality remained significant ($p<0.001$) when adjusted for these confounding factors. Therefore these factors do not influence the relationship between age and all-cause mortality in these cases, and thus age is an independent risk factor for all-cause mortality.

Breast cancer specific mortality remains significant between the age groups after adjustment. However, there is an increase in risk of breast cancer specific mortality in the screening group between the adjusted and unadjusted. The HR is 0.39 (95% CI; 0.31-0.49) in the unadjusted analysis and 0.69 (95% CI; 0.51-0.95) in the adjusted screening group (*Table 4.3)*; which suggests that one of the factors adjusted for contributes to the increased survival in these women. When the analysis was adjusted, women in the pre-screening group have a lower risk of breast cancer specific death than the screening group (40% vs 31%). This observed difference between the two may be due to stage at diagnosis, which is lower in the screening women, or deprivation.

Unadjusted Analysis	**All-Cause Mortality**		**Breast Cancer Specific Mortality**		**Disease Free Survival**	
	HR (95% CI)	*P value*	*HR (95% CI)*	*P value*	*HR (95% CI)*	*P value*
>70yr	1.00	<0.0001	1.00	<0.0001	1.00	<0.0001
<50yrs	0.28 (0.22-3.4)*		0.60 (0.46-0.79)*		1.00 (0.78-1.30)	
50-69yrs	0.25 (0.22-0.30)*		0.39 (0.31-0.49)*		0.57 (0.46-0.72)*	
Adjusted Analysis						
	HR (95% CI)	*P value*	*HR (95% CI)*	*P value*	*HR (95% CI)*	*P value*
>70yr	1.00	<0.0001	1.00	0.01	1.00	0.53
<50yrs	0.31 (0.24-0.41)*		0.60 (0.42-0.85)*		0.85 (0.62-1.18)	
50-69yrs	0.39 (0.31-0.48)*		0.69 (0.51-0.95)*		0.86 (0.64-1.15)	

Table 4.3 - Cox regression analysis by age for all three outcomes.
*** Significant.**
[a] Adjusted for deprivation, size, grade and nodal status.

The relationship between age and disease free survival lost its significance when the analysis was adjusted for the above factors. Women in the screening group no longer had a decreased risk of recurrence when compared to those in the post-screening group HR = 0.86 (95% CI 0.64-1.15; p = 0.53) (*Table 4.3*). To further analyse the influence of the factors adjusted for on this relationship between disease free survival and age, each confounding factor was removed from the analysis individually and the effect on the HR significance observed. This showed that when information on stage (tumour size, grade and nodal status) was removed from the analysis then the significant relationship returned. This suggests that deprivation and age are independent risk factors, but there is a difference in stage between these women which accounts for the difference in disease free survival. This may relate to women of screening age being diagnosed with lower stage cancers as they are picked up earlier than women of pre- and post- screening age.

3.5 Socioeconomic Status and Survival

Socioeconomic status was calculated using the Carstairs index(288), which separated the women into 10 groups depending on postcode at diagnosis. The Carstairs index was chosen in this instance as it combines 4 indicators of disadvantage to give an indication of deprivation and it was based on the 2001 census data, which is relevant as this data was collected between 2000 and 2004. However it was found that in some of the deciles there were few participants and even fewer events, therefore it was not suitable to analyse the data in these ten groups. For this reason these ten groups were divided into three groups: affluent (n=590 women), moderate (n=719 women) and deprived (n=369 women). This demonstrates that more women with a higher SES are diagnosed with breast cancer, than those with a lower SES. Upon comparison it was observed that there was a significant difference in survival between groups for all 3 outcomes (log rank tests – all-cause p = 0.002; breast cancer specific p = 0.007; disease free survival p = 0.035)(*Figure 4.6*). In each of the outcomes there was little difference observed between women in the affluent and moderate groups (*Table 4.1*), but women in the deprived group had consistently poorer outcomes. For example, women in the affluent and moderate SES groups had a cumulative 5 year breast cancer survival of 84% (+/-2%) and 85% (+/-1%) respectively. However, women in the deprived group had a 5 year survival of 75% (+/-2%) (*Table 4.1*). In general, it was noted that there was a 10% difference in 5 years survival between deprived women and those in the higher SES groups across all outcomes (*Figure 4.6*). This relationship is perhaps more pronounced in the all-cause mortality outcome, which suggests that women in the most deprived group are more likely to die but that this death may not necessarily be a breast cancer specific death.

This analysis was then replicated using the women in the top 3 and bottom 3 deciles; to negate any bias which may have been caused by division into 3 SES groups (*Figure 4.6ii*). This confirmed the results of the previous analysis with a significant difference on Kaplan-Meier log rank testing between the most affluent and most deprived being observed across all three outcomes in analysis (log rank tests – all-cause p = 0.002; breast cancer specific p = 0.003; disease free survival p = 0.016) (*Figure 4.6ii*).

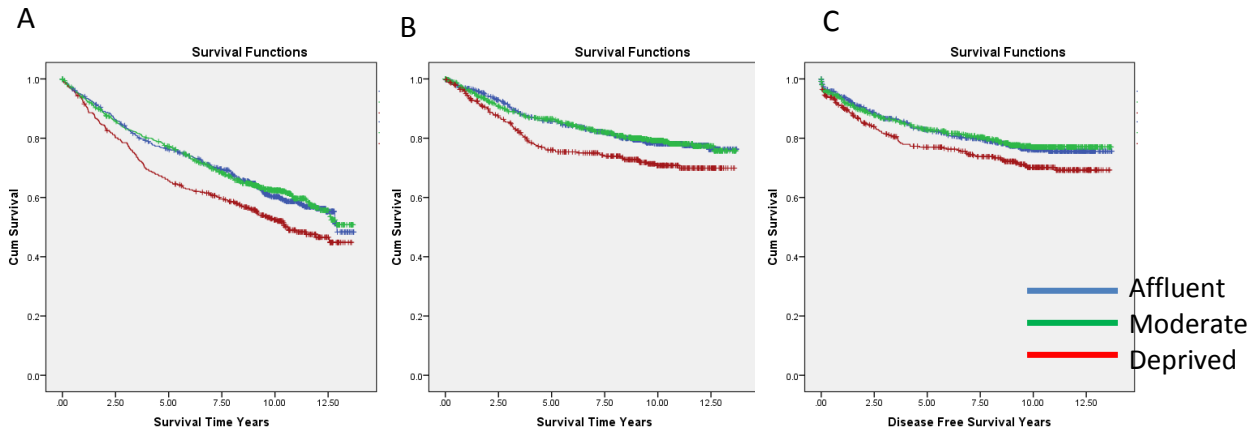
As with age this analysis was taken further using both univariant and multivariant cox regression analyses to look at the risk associated with deprivation and breast cancer outcome. The univariant analysis of the three SES groups found that women in the affluent group had a 31% decreased risk of breast cancer specific death compared to those in the most deprived group (HR = 0.69; 95% CI 0.52-0.90; p = 0.007)(*Table 4.4*). These women also hava a 25% decreased risk of all-cause mortality compared to the most deprived women (HR = 0.75; 95% CI 0.62-0.9; p = 0.004). Women in the moderate SES category had similar survival to those in the affluent group across all outcomes (all-cause HR = 0.74; breast cancer specific mortality HR = 0.68, and disease free survival HR = 0.73). Disease free survival was also significantly (p = 0.036) better in women who fell into the affluent and moderate categories when compared to deprived women (*Table 4.4*).

When these results were adjusted for age, tumour size, grade and nodal status there was a change in the significance of these relationships. The significant difference between affluent and deprived groups was lost across all outcomes; however a significant relationship between the moderate and deprived groups remained (*Table 4.4*). To further analyse these results, as with the analysis for age, the confounding factors were removed one by one from the analysis and the results observed. When tumour size, grade and nodal status were removed from the analysis the significant difference between the affluent and deprived groups returned. This suggests that

there is a difference between tumour stage at diagnosis, which influences the difference in survival associated with deprivation.

The multi-variant cox regression was also carried out on the top and bottom deciles. The adjusted analysis failed to find any significant survival between affluent and deprived women for breast cancer specific mortality (HR = 0.74; 95% CI: 0.54-1.02; p=0.064) or disease free survival (HR = 0.86; 95% CI: 0.65-1.16; p=0.33)(*Table 4.4*). Further analysis demonstrated that the reason for the loss of the significant difference

i) Deprivation Categories – Affluent, Moderate, Deprived



ii) Deprivation 1-3 versus 8-10

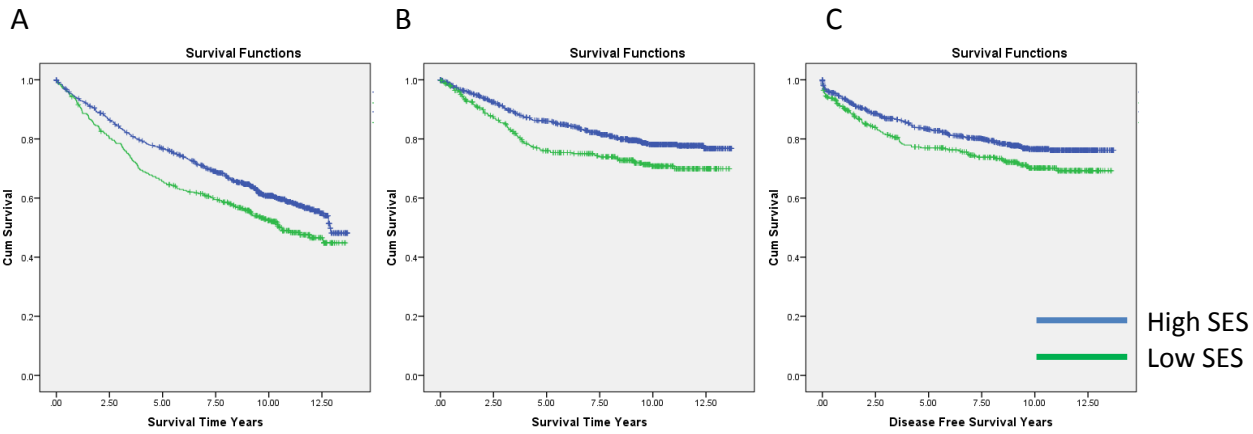


Figure 4.6 Deprivation analysis Kaplan-Meier plots. i) Deciles grouped in to the categories affluent (1,2), moderate (3-7) and deprived (8-10). All three outcomes A) All-cause mortality, B) Breast cancer specific mortality and C) Disease free survival has significant results on log rank testing. ii) The final group are the top deciles versus the bottom deciles, all three were significant on Log rank testing.

was due to the inclusion of staging information; once again demonstrating that stage is responsible for the difference observed between women with a high and low SES (*Appendix 4*).

Unadjusted Analysis	**All-Cause Mortality**		**Breast Cancer Specific Mortality**		**Disease Free Survival**	
	HR (95% CI)	*P value*	*HR (95% CI)*	*P value*	*HR (95% CI)*	*P value*
Deprived	1.00	0.003	1.00	0.007	1.00	0.036
Affluent	0.75 (0.62-0.91)*		0.69 (0.52-0.90)*		0.75 (0.58-0.98)*	
Moderate	0.74 (0.61-0.89)*		0.68 (0.53-0.89)*		0.73 (0.56-0.94)*	
Adjusted Analysis[a]						
	HR (95% CI)	*P value*	*HR (95% CI)*	*P value*	*HR (95% CI)*	*P value*
Deprived	1.00	0.15	1.00	0.01	1.00	0.037
Affluent	0.80 (0.62-1.03)		0.86 (0.62-1.20)		0.98 (0.72-1.34)	
Moderate	0.70 (0.55-0.89)*		0.62 (0.45-0.85)*		0.72 (0.53-0.97)*	
Adjusted Analysis[a]						
	HR (95% CI)	*P value*	*HR (95% CI)*	*P value*	*HR (95% CI)*	*P value*
1-3 Vs 8-10	0.75 (0.59-0.95)*	0.017	0.74 (0.54-1.02)	0.064	0.86 (0.65-1.16)	0.33

Table 4.4 - The Cox regression analysis by deprivation category for all three outcomes.
*** Significant.**
[a] Adjusted for age, size, grade and nodal status.

The significant difference between the risk of all-cause mortality between the two SES groups after adjusting for potential co-founding factors remained – HR = 0.75 (95% CI: 0.59-0.95; p = 0.017). Therefore, women in the affluent group have a 25% decrease in risk of dying of any cause in comparison to women in the most deprived groups. This suggests that deprivation is an independent risk factor for all-cause mortality in women diagnosed with breast cancer.

4. Discussion

4.1 Overall Trends in Survival

The results of this analysis on a Tayside cohort confirmed the associations between tumour size, grade, nodal status and receptor status with breast cancer outcome. In addition, this has highlighted the relationship between survival age, deprivation and year of diagnosis for the three outcome measures used.

An earlier study by Twelves et al(283) found the 5 year survival of Scottish women diagnosed in 1987 to be 70.9% (95% CI; 68.6-73.1). A later study published in 2001 showing the 10 year survival in the same cohort found the survival to be 52.7% (95% CI; 50.3-55.1)(275). A third study which aimed to compare the survival of this 1987 cohort to that of a 1993 cohort found that women diagnosed in 1993 had over a 10% increase in 8 year survival from 57.4% to 68.3%(289). This highlights that there has been a trend towards increasing survival in the past, which has been predicted to be equivalent to an annual decrease in mortality of 1.8% between 1997 and 2006 in a Spanish population(290).

To allow comparison to other Scottish studies and EUROCARE-5, the all-cause mortality analysis was performed with all inoperable cases excluded. This demonstrated that for women diagnosed in Tayside between 2000 and 2004 there was a 5 year cumulative survival for all-cause mortality in operable cases of 80% (+/- 1%) and 10 year survival was 66% (+/-1%). This is a 10% improvement in 5 year survival when compared to women diagnosed in 1987 and a 13% improvement in 10 year survival(283). When comparisons are drawn to the 8 year survival of both the 1987 and 1993 cohorts, which were 57.4% and 68.3% respectively(289), the 8 year survival was 72% (+/-1%); which shows there has been further improvements made in the survival of operable cancers over the past decade.

EUROCARE-5 found the UK and Ireland mean 5 year all-cause mortality survival to be 78.5% for operable cases, which was lower that the European mean of 82%(285). In this Tayside cohort survival was higher than the UK and Ireland average as predicted by EUROCARE and more comparable with the European average at 80%.

The 5 year survival for breast cancer specific mortality in previous literature has been found to be 81.6% in Scottish women diagnosed between 1986 and 2000(276). In this study it was found that the 5 year breast cancer specific survival was 82%, which indicates that there has not been any significant improvement in breast cancer specific survival over the last decade. This is in agreement with the lack of improvement noted when analysing the cases dependent on year of diagnosis, which failed to show any significant relationship (*Table 4.2*). However, there was an indication that there may be a trend towards improving survival for breast cancer specific mortality; women diagnosed in 2000 had a 28% increase in risk of dying of breast cancer compared to those diagnosed in 2004. This is consistent with published literature which found an estimated percentage change in breast cancer specific mortality to be 2% between 1993 and 2003(291). However, the failure of the current analysis to reach significance may reflect the relatively small numbers of patients diagnosed each year in Tayside and that only a short time period was investigated (2000-2004).

This analysis also demonstrated that there was a 10% difference between all-cause mortality and breast cancer specific mortality survival. This suggests that women diagnosed with breast cancer are more likely to die of something other than breast cancer between 5 to 10 years after diagnosis. All-cause mortality analysis shows that survival continues to fall from 70% to 56% between 5 and 10 years, but in the breast cancer specific mortality outcome survival begins to level out only falling from 82% to 76% over the same time period (*Figure 4.1*). Disease free survival showed a similar relationship to breast

cancer specific survival, with the majority of recurrence events happening in the first 5 years after diagnosis.

4.2 Deprivation and Survival

Inequalities in breast cancer survival are a well-documented phenomenon, with women who are from the most deprived SES groups having consistently worse outcomes(272,273,275,276,284). In this cohort women in the most deprived group had a consistently poorer outcome regardless of the outcome measure being investigated – all-cause mortality, breast cancer specific mortality and disease free survival. When the analysis was adjusted for confounding factors such as stage and age the significant difference between the affluent and deprived group was lost. This demonstrated that in this cohort at least part of the survival difference was related to tumour stage at diagnosis. This is a finding which has been previously suggested by other published data, which predicted that stage accounted for 28% of the deprivation gap(278). That study found that women in the lowest SES had a 23% increase in risk of breast cancer specific death(278), whereas in this analysis the risk was found to be 31%.

To remove bias that may have been caused by the division of Carstair deciles into 3 groups a second analysis was carried out comparing the top and bottom SES categories. The results of this analysis were the same as that of the above analysis. With the exception of all-cause mortality which remained significantly different between the two groups once adjusted for stage and age. Women in the most affluent SES group had a 25% decreased risk of all-cause mortality, the reason for this may relate to differences in health or social factors between the deprived and affluent populations.

When assessing survival in the three groups the significant difference remained between the moderate and deprived groups after adjustment for stage and age. The reason and significance of this finding is unknown, but it may relate to the division of the data into 3

groups as the moderate group was the largest. Other potential explanations of this finding are the measure of deprivation used, awareness in this population group or biological factors.

The survival analysis in this cohort demonstrates that there is a disparity in survival between women in the least and most deprived SES groups. This relationship was found to be related to stage when breast specific mortality risk was being investigated, as observed by other studies(278,284). This difference in stage at presentation may relate to multiple factors such as breast awareness, access to health care or participation in the screening programme. However, this difference in survival may have other contributing factors out-with the scope of this analysis, such as tumour biology, genetics(273,279), or awareness in the community(282).

Unfortunately, there was no information on other environmental prognostic factors or any risk factors collected. It may have been interesting to further investigate the effect of these factors in this group of participants. In the future it would be of great interest to see if any of the other environmental or genetic risk factors, identified in this thesis, interact with the impact SES has on outcome. This may be something to pursue in the future, but would require the inclusion of an extensive questionnaire and genotypes in a large group. The current VPH-PRISM project, which is recruiting in Dundee has an in depth questionnaire; this questionnaire includes information on all the environmental risk and prognostic factors deemed to have strong supporting evidence by this thesis. This cohort will therefore allow a more in depth analysis of these factors on survival after a period of follow up.

This analysis demonstrates that deprived women have a worse outcome than women with a high or moderate SES (*Figure 4.6)*, this effect was seen regardless of how SES was defined. This association was lost when the analysis was adjusted for staging information; therefore poorer outcome in deprived women may be in part due to stage at diagnosis. In

addition there are more women with a higher SES diagnosed with breast cancer in Tayside (affluent 35%, deprived 21%) this supports that a high SES is associated with increased breast cancer risk.

4.3 Conclusion

One of the strengths of this analysis is the data-set which is extremely complete and consistent. The reasons for this include that the treatment was carried out by a single multidisciplinary team, the breast tissue and lymph node samples were analysed by a single team of pathologists. Patients were followed up using both medical notes and death certificates, which meant that an accurate cause of death was identified in all patients. Criticism may arise with regards to the inclusion of DCIS, LCIS and inoperable cancers in the analysis; however, this was done to give a clear indication of survival when all cases were included and not those picked for favourable characteristics. Additionally, there may be questions as to why the dataset was split into the 3 risk groups – the reason for this was to try and split the participants as equally as possible. In an attempt to control for this, the deprivation was analysed a second time to compare the top and bottom deciles. This second analysis confirmed the results observed in the initial analysis, finding the same association between deprivation and poor outcome.

These results from this cohort demonstrate an increasing survival in Scotland and Europe, with the majority of breast cancer deaths and recurrences happening within the first 5 years after diagnosis. These results also confirm that, as discussed in the previous chapter deprivation is associated with a poorer outcome. Stage was identified as contributing to poorer breast cancer specific outcomes, but not to all-cause mortality. The reason for this relationship between breast cancer and deprivation requires further research to elucidate why the two are linked. However as previously demonstrated breast cancer risk and

prognosis are determined by a complex mixture of environmental, reproductive and genetic factors and, so to understand risk and prognosis requires a holistic approach.

Conclusion

When beginning this project there were several key questions which required answers in order to provide a different perspective on breast cancer pathogenesis; a different perspective which is more holistic and incorporates different elements of the disease. Prior to being able to view this heterogeneous disease in a holistic manner, the information on the individual elements had to be assessed. Therefore, a series of literature reviews were undertaken to gather the facts from the wealth of published literature available to come to conclusions about what contributes to breast cancer risk and survival.

There have been a large number of studies published which investigate the many different aspects of epidemiological breast cancer risk. From the current literature review it has been concluded that reproductive factors such as age at menarche, age at FFTP, parity, current HRT use, birth control use and age at menopause all contribute to breast cancer risk; most likely through a hormonally driven mechanism, which relates the developmental stage of the breast tissue. In addition, other environmental risk factors exist including a high post-menopausal BMI, smoking, exposure to ionising radiation, alcohol consumption, ethnicity, socioeconomic status and mammographic density which all increase breast cancer risk via variety of mechanisms.

Genetics also influence breast cancer risk; there are a combination of high and low risk genetic changes which contribute to the risk of developing breast cancer via a variety of mechanisms. However, little is understood about how some genetic changes alter risk. The majority of breast cancer is a combination of various risk factors which add together to reach a disease threshold (50).

Additionally there is a growing amount of research being carried out on gene-environment interactions in breast cancer and how these may modify risk. For example, there is emerging evidence that the risk associated with *FGFR2* polymorphisms may differ depending on age at menarche, parity and a BMI>25 in post-menopausal women(226). This is particularly interesting as *FGFR2* and the other implicated risk factors are all thought to exert their effect on risk via hormonal mechanisms. It could therefore be hypothesised that these environmental and genetic risk and prognostic factors all work within the same cellular pathways. These gene-environment interactions may exist as they target common pathways and by identifying these pathways more can be learned about breast cancer pathogenesis. Gene-environment interactions also provides a framework of thinking to allow new breast cancer risk factors, both genetic and environmental, to be discovered.

The work on gene-environment interactions is in its early stages with research failing to come up with conclusive evidence implicating a particular set of interactions. However as of yet there seems to be a failure to use the knowledge about individual risk factors to target the research into gene-environment interactions, as suggested above. Pathways which appear to underlie the mechanism of risk in multiple genetic and environmental risk factors are DNA damage and repair, cell cycle control, apoptotic processes, oestrogen signalling, the microenvironment and breast tissue development pathways. Many of the factors come under more than one of these headings and they all have different roles to play in these wide biological process headings (*Figure 5.1*). However, as stated during the genetics chapter, some genetic factors are also known to bind and interact; for example, ATM is needed to activate CHEK2.

Another interesting point is that some of the environmental factors which influence risk also influence prognosis; these include BMI, weight loss, HRT use, and socioeconomic status. However, some factors were associated with better outcome despite being

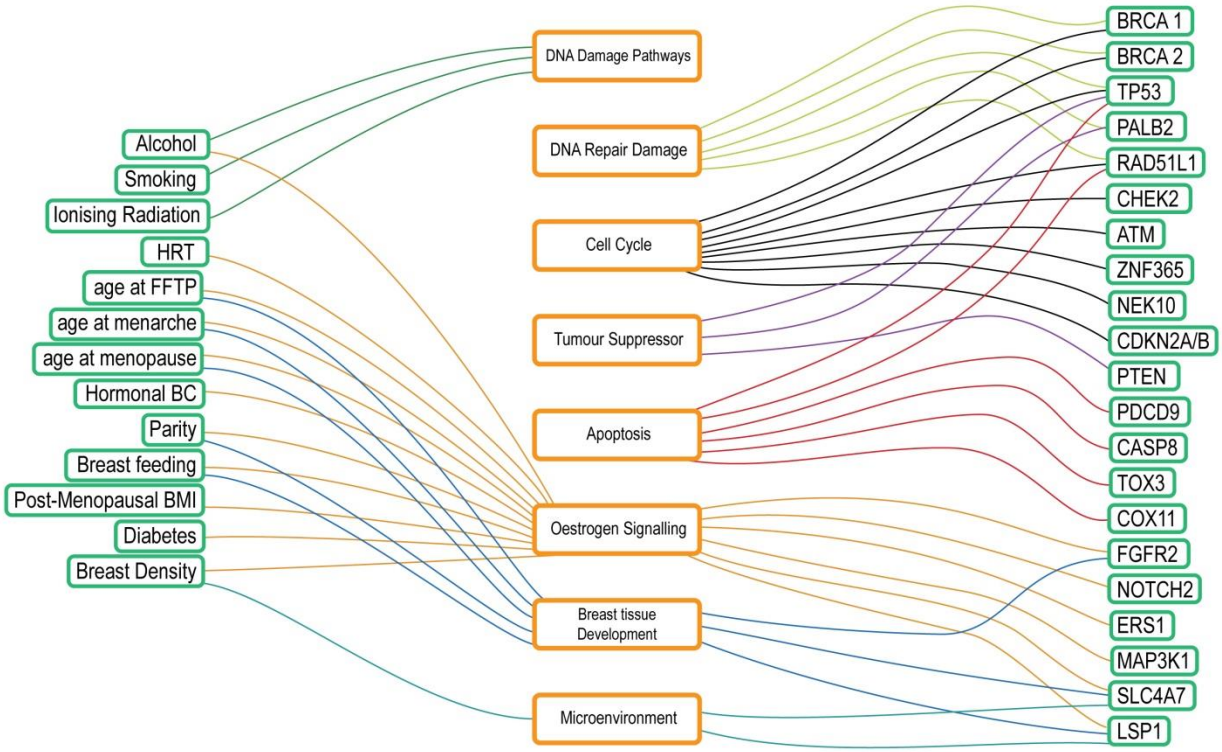


Figure 5.1 - This is a diagrammatic representation of the biological mechanisms which are found to be associated with breast cancer risk. In the centre are the biological mechanisms highlighted as having an involvement in breast cancer pathogenesis. On the left are the environmental risk factors and on the right are the genetic risk factors. A line connects the risk factors to the biological mechanism(s) which are thought to underlie how they alter breast cancer risk.

associated with increased risk – such as high SES and HRT. High BMI on the other hand was associated with an increased risk and poorer prognosis. So once again there are common environmental factors involved in these processes and this may be due to common mechanisms at play. However, it is also possible that prognostic factors are at least in part working by a different mechanism to affect outcome. When deprivation was investigated in the Tayside cohort it was consistently associated with a poorer breast cancer specific outcome. In part, this poorer prognosis was due to the effect of tumour stage, as those from a more deprived area appeared to have higher stage tumours. The analysis also confirmed that ER negative, PR negative, lymph node involvement, older age, larger size and higher grade tumours were all associated with poorer outcome, as expected. As there

was no information on other environmental prognostic factors collected in this cohort analysing these in greater detail was unfortunately not possible. In the future, the collection of information on further epidemiological parameters (reproductive risk factors, BMI, as well as SES) and genetic information on patients diagnosed with breast cancer would allow a study of their influence on prognosis and any gene-environment interactions in this context – the VPH-PRISM study may allow this.

Another element which has not been covered in depth but is important in tumour behaviour and is potentially influenced by genetics, the environment and their interactions is the microenvironment(292). This has already been touched upon when discussing genetic risk factors, as some of the low penetrance polymorphisms associated with risk - SLC4A7 and LSP1 - are involved in the regulation of the microenvironment. The microenvironment itself consists of extra-cellular matrix and stromal tissue; this stroma consists of fibroblasts, adipocytes, pre-adipocytes, blood vessels, and inflammatory cells(293). The stromal tissue and epithelial cells interact, and this interaction plays a role in the neoplastic processes of initiation, progression, invasion and angiogenesis(294)291).

Breast stroma also plays a crucial role in the normal development of breast tissue. This developmental process in women has a hormonally driven element and is also triggered by certain reproductive events in a woman's life such as first full term pregnancy and breastfeeding. This is a process which has been implicated in both environmental and genetic breast cancer risk. The stroma is thought to provide the signals which encourage the breast epithelium to develop to maturity and this, in turn, provides a link between the stromal tissue and reproductive risk factors associated with breast cancer development. If or how these two factors interact is unknown, but it provides an interesting area of research. Additionally stromal factors may be linked to the added risk associated with increased breast density; one suggested reason as to why high density equates to high risk

is that there is an unknown factor which increases risk. This unknown factor may well be a stromal factor, which increases risk and has caused a high breast density due to its interaction with surrounding tissues causing a higher density of epithelial tissue and collagen(296).

Previous studies have found there to be differences in expression between normal and tumour associated stroma. These differences have been hypothesised as being involved in these tumour-stromal interactions which could be potential pharmacological targets in the future(297,298). Some examples of genes up-regulated in tumour associated stroma were *GRAM1* and *INHBA*, both of which are members of the *TGFβ* family. Other genes were found to be down-regulated in the tumour associated stroma such as *WIF1* and *SFRP1,* both members of the WNT signalling pathway(296).

Therefore it can be hypothesised that the genes up and down regulated in the reactive stroma and tumour tissue could include those already identified as risk factors and new novel genes which are part of the common pathways identified as involved in breast cancer risk and prognosis. To test this hypothesis would require sampling breast tissue at three locations (tumour, peri-stromal area and normal stroma), RNA extraction and then microarray analysis. A proof of concept experiment was carried out as detailed below in Appendix 5; however extraction of RNA from formaldehyde fixed paraffin embedded tissue (FFPE) yielded a suitable quantity of RNA products, which were unfortunately of too poor a quality to carry out any further experiments. It was therefore concluded that a future experiment with fresh tissue would be the most suitable experimental methodology, but due to time constraints this will be out-with the scope of this current thesis (Protocol in Appendix 5).

This thesis aimed to demonstrate that there are common biological mechanisms which underlie many of the genetic and environmental risk and prognostic factors that have been

associated with breast cancer. There is not a single process involved and each risk factor may be involved in multiple pathways to contribute to risk or outcome. Gene-environment interactions also exist and act to modify breast cancer risk, but there is still work to be done in this area to provide clarity. A single factor can also act differently on risk and prognosis, such as deprivation which was shown to influence outcome in a novel analysis. All of the above demonstrate that breast cancer is a complex heterogeneous disease, which can be best understood, not by looking at individual elements but by taking what is known about these elements and looking at them in a holistic way to inform future hypotheses and study.

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APPENDICES

Appendix 1. *Search Terms Used in Medline for Genetic Risk Factors*

Genetic Risk Factor	Search Terms : “Breast Neoplasms AND”
BRCA1	BRCA1
BRCA2	BRCA2
ATM	ATM
CHEK2	CHEK2
TP53	p53
PTEN	PTEN
FGFR2	FGFR2
TNRC9 (TOX3)	TNRC9
NOTCH2	NOTCH2
ZNF365	ZNF365
RAD51L	RAD51L
ESR1	ESR1
CASP8	CASP8
NEK10, SLC4A7	NEK10, SLC4A7
MAP3K1	MAP3K1
CDKN2A/B	CDKN2A/B
LSP1	LSP1
COX11	COX11

Appendix 2. *Search Terms Used in Medline for Environmental Risk Factors*

Environmental Risk Factor	Search Terms : “Breast Neoplasms (aetiology and epidemiology) AND”
Age at menarche and menopause	“Menarche” OR “Menopause”
Age at FFTP and parity	“Parity” or “Full term Pregnancy”
Breastfeeding	“Breastfeeding”
Hormone replacement therapy and hormonal birth control	“Hormone Replacement AE/ Contraceptives, Oral, Hormonal AE”
Family History	“Familial”
BMI	“Body Mass Index”
Diet	“Diet” OR “High Fat Diet”
Alcohol Consumption	“Alcohol Drinking/AE”
Smoking	“Smoking”
Environmental Exposures	"Environmental Exposure"
Ionising Radiation	“Radiation”
Ethnicity and SES	“Ethnicity” OR “Socioeconomic Status”

Appendix 3. *Search Terms Used in Medline for Prognostic Factors*

Environmental Risk Factor	Search Terms : “Breast Neoplasms AND Prognosis AND”
Hormone replacement therapy and hormonal birth control	“Hormone Replacement AE/ Contraceptives, Oral, Hormonal AE”
BMI	“Body Mass Index”
Diet	“Diet” OR “High Fat Diet”
Alcohol Consumption	“Alcohol Drinking/AE”
Ethnicity and SES	“Ethnicity” OR “Socioeconomic Status”
Physical Activity	“Physical Activity” OR “Exercise”

Appendix 4. *Survival and Deprivation*

This table demonstrates the effect of removing each component of staging from the adjusted analysis and its effect on significance.

	All Cause		**BrCa Specific**		**Disease Free Survival**	
	HR	*P value*	*HR*	*P value*	*HR*	*P value*
Nodes removed from analysis						
Deprived	1.00		1.00		1.00	
Affluent	0.77*	0.05	0.80	0.187	0.92	0.585
Moderate	0.72*	0.008	0.62*	0.004	0.72*	0.034
Size removed from analysis						
Deprived	1.00		1.00		1.00	
Affluent	0.77*	0.016	0.79	0.104	0.895	0.431
Moderate	0.685*	0.0003	0.63*	0.001	0.73*	0.021
All Staging info removed from analysis						
Deprived	1.00		1.00		1.00	
Affluent	0.735*	0.002	0.69*	0.007	0.77*	0.05
Moderate	0.68*	0.00005	0.66*	0.002	0.72*	0.013

***Significant p <0.05**

Appendix 5. *Proof of Concept Molecular Analysis*

Fixed Tissue RNA Extraction

As covered in the conclusion to further explore the relationship between the pathways involved in breast cancer the expression of genes which have been previously implicated breast cancer risk can be investigated in breast stroma, reactive stroma and breast cancer. One of the simplest ways to retrieve samples of stromal tissue would be to take cores from fixed tissue blocks. However there is poor evidence that good quality RNA can be extracted from formaldehyde fixed paraffin embedded (FFPE) tissue. It was for this reason that prior to starting tissue collection from participants for this study a proof of concept experiment was run with samples from Tayside tissue bank, to examine the quantity and quality of any RNA that could be extracted.

Methods

Samples were selected from Tayside tissue bank with the assistance of Dr Purdie, who selected three matched breast tumour and normal breast stroma from the tissue bank catalogue. From the tissue blocks Dr Purdie marked an area from which the 1mm core was to be taken, chosen for their typical cellular characteristics. 1 mm cores were taken from these sites to allow for isolation of total RNA from the FFPE tissue using the Purelink FFPE Total Isolation Kit by Invitrogen. There was a second elution taken of all samples during the washing stages of the extraction to ensure all the RNA extracted was collected. These samples underwent analysis separately as the second elution samples in all later experiments. This was followed by a DNase treatment using an Invitrogen kit on the extracted RNA. Each sample was named after the block from which the sample came followed by N or T for normal and tumour respectively (IC_N, IC_T, IE_T, IG_T, IL_N and IN_N).

The treated RNA was then quantified using the Invitrogen Qubit 2.0 Fluometer and the Qubit high sensitivity RNA assay using 2 µL of RNA per assay. The quality of the RNA was also assessed using the Aligent R6K Screen Tape System Tape Station and the high sensitivity R6K kit.

Two kits were used to allow comparison for the reverse transcription step: Reverse Transcription System by Promega and the High Capacity cDNA Reverse Transcription kit by Applied Biosystems. The cDNA produced was stored at -20 ˚C, to do test RT-PCR experiments on at a later date if the RNA product was considered of a high enough quality.

Any remaining RNA was stored at -80˚C for further use if necessary. All practical work was carried out with the assistance of Dr Andrew Cassidy and Dr Desiree Rutschow in the Human Genetics Laboratory Ninewells Hospital.

Results

Samples came from three cases and for each case there was a tumour sample and matched normal sample. These cases were case 1 IC_N and IC_T; case 2 IN_N and IE_T; and case 3 IL_N and IG_T. After the RNA has been extracted the Invitrogen Qubit 2.0 Fluorometer with the high specificity RNA kit was used to try and quantify the amount of RNA present at a 1:100 dilution. The results are shown in the table below (*Table 5.1*), and it is clear that some samples in the 2nd elution failed to produce a measurable concentration of RNA. Otherwise there was a wide range of results from 2.8 ng/µL to 86.0 ng/µL. These results indicate that from the fixed tissue RNA could be extracted, however the quantity was extremely variable. This may relate to the cellular content of the samples as the concentrations of RNA in normal stroma was considerably less than that of the tumour tissue, which is to be expected.

Case	Sample	Elution 1 (ng/µL)	Elution 2 (ng/µL)
1	IC_N	2.80	-
	IC_T	14.20	4.72
2	IN_N	5.92	-
	IE_T	86.00	5.51
3	IL_N	16.20	2.87
	IG_T	50.00	20.40

Table 5.1 – The concentration of the extracted RNA as calculated by the Invitrogen Qubit 2.0 Fluorometer for both the first and second elution. This shows there was a large range of RNA concentrations extracted.

Once the RNA samples were quantified it was important to then check the quality of the RNA extracted, to do this the Aligent R6K Screen Tape Station was used. This demonstrated that the extracted RNA was highly degraded and of poor quality failing to produce the desired bands at 18s and 28s.

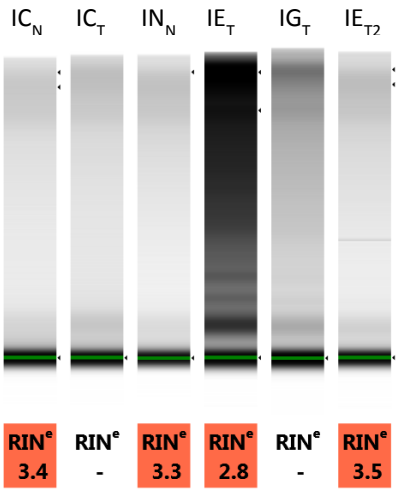

Figure 5.1 - The results of the Aligent Tape Station which shows the extracted RNA is highly degraded; failing to produce bands at 18 and 28s. The accompanying RIN values are also shown.

A second tape station was run for certain samples where there was still RNA for analysis, namely IC_N, IC_T, IN_N, IE_T, IG_T and IE_{T2} (2nd elution). These samples were run at a lower concentration of 1:20 dilution to try and further assess the quality of RNA. Once again there was a failure to produce bands at 18s and 28s (*Figure 5.1*). Additionally the RIN (RNA integrity number) values obtained were below the standard of 4 which is required for microarray analysis. For these reasons it would not suitable for further use in either an RT-PCR or microarray analysis.

Conclusion

This therefore confirmed that though there was a high quantity of RNA extracted from some of these samples (*Table 5.1*), it was extremely degraded (*Figure 5.1*) and unusable. Additionally the optimum concentration of RNA needed for microarray is approximately

100-300 ng per 3 µL, therefore adequate concentrations of RNA were only obtained in 2 of the above samples. The minimum RIN value requirement for microarray is also 4 and all of the samples failed to meet this quality measure across both analyses. This meant that though a simpler way of obtaining samples, RNA extracted from fixed tissue was not suitable for the purpose of this experiment as it failed to produce a consistent product which was of adequate quantity or quality. Therefore the decision was taken to explore alternative options for fresh tissue sample collection. A second option is to use fresh frozen samples stored in an RNA preservative. These samples shall allow the extraction of RNA, which is suitable for variety of methods of genetic analysis. This will allow any alterations in expression between normal stroma, reactive stroma and breast tumour to be identified and analysed.

Revised Protocol for Tissue Collection

- Four suitable candidates shall be selected to have tissue samples for the purpose of genetic analysis.
    - Inclusion criteria:
        - Undergoing mastectomy,
        - ER and PR positive
        - HER2 negative
        - DNST
        - Core grade 2 or3
        - BIRADS breast density 2-4.
    - Exclusion criteria
        - Treated with neoadjuvant chemotherapy.
- Potentially eligible participants identified at MDM

- Pre-prepared vials of RNA later labelled with study number, and T for tumour PT for peri-tumoural and N for normal stroma.
- On the day of surgery vials will be collected from the fridge and take them to the breast imaging until at a previously specified time.
- Specimen is collected from the operating theatre, and taken to the breast imaging unit for sample collection.
- 14G core biopsy samples - normal stroma, reactive stroma and tumour, identified using shear wave ultrasound elastography
    - A shear wave image of each sample site shall also be recorded.
- Samples will be delivered back to the same fridge.
    - The tissue (in RNA later) is then transferred into an appropriate fridge at 4°C until such a time (up to 5-7 days) as it can be transferred to a -20°C freezer (L6012) (to be stored for up to 1 month), or processed.
- Using a well-established manufactured kit, RNA shall be extracted from these samples to ensure the best quality RNA is stored for further use at -80˚C in a freezer or will be immediately converted into cDNA as it is more stable – this will be dependent on the next steps/microarray procedure.
- Quality controls of the material will include – Aligent Tape station (and Invitrogen Qubit 2.0 Fluorometer).
- These samples shall be used as appropriate for differential gene reaction studies such as microarray, allowing expression comparison between tumour, reactive and normal stroma